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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Review

Biofertilizer, a way towards organic agriculture: A review

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Fertilizers supply essential plant nutrients, mainly nitrogen (N), potassium (K) and phosphorous (P). These fertilizers increase the yield of the crop but they cause several health hazard. Due to the several health hazard, consumer preferences shift towards the use of the organic food grown without use of any chemical. In recent years, biofertilizers have emerged as an important component for biological nitrogen fixation. They offer an economically attractive and ecologically sound route for providing nutrient to the plant. Biofertilizers are low-cost renewable source of nutrient that supplements the chemical fertilizer. Biofertilizers gained importance due to its low cost amongst small and marginal farmer.

Key words: Biofertilizer, isolation, mass multiplication, N_2 fixers, plant growth promoting rhizobacteria (PGPR), organic agriculture.

INTRODUCTION

The term biofertilizer, represent everything from manures to plant extracts. “Biofertilizers” are those substances that contain living microorganisms and they colonize the rhizosphere of the plant and increase the supply or availability of primary nutrient and/or growth stimulus to the target crop. There are numerous species of soil bacteria that colonize mainly in the rhizosphere of plants. These bacteria are collectively known as plant growth promoting rhizobacteria (PGPR). Some PGPR promote the growth by acting as biofertilizer. Microorganisms mainly nitrogen fixer, phosphate solubilizer and mycorrhizae are the main sources of biofertilizer. The microorganisms used for the biofertilizer are bacteria of *Bacillus, Pseudomonas, Lactobacillus*, photosynthetic bacteria, nitrogen fixing bacteria, fungi of *Trichoderma* and yeast. Biofertilizers have shown great potential as a, renewable and environmental friendly source of plant nutrient. Biofertilizers are ready to use and used as a live formulation of beneficial microorganisms, when it amended to seed, root or soil, it mobilizes the availability and utility of the microorganisms and thus improves the soil health. In general, bio-fertilizers are microbial preparations containing living cells of different microorganisms which have the ability to mobilize plant nutrients in soil from unusable to usable form through biological process. Bio-fertilizers are used in live formulation of beneficial microorganism which on application to seed, root or soil, mobilize the availability of

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Table 1. Carriers.

Form	Liquid	Powder	Granular
Appearance	Without strange smell	Brown	Brown
Fast-growing *Rhizobium*	>0.5 x 10^9/ml	>0.1 x 10^9/g	>0.1 x 10^9/g
Slow-growing *Rhizobium*	>1.0 x 10^9/ml	>0.2 x 10^9/g	>0.1 x 10^9/g
N fixing bacteria	>0.5 x 10^9/ml	>0.1 x 10^9/g	>0.1 x 10^9/g
Si bacteria	>1.0 x 10^9/ml	>0.2 x 10^9/g	>0.1 x 10^9/g
Organic P	>0.5 x 10^9/ml	>0.1 x 10^9/g	>0.1 x 10^9/g
In organic P	>1.5 x 10^9/ml	>0.3 x 10^9/g	>0.2 x 10^9/g
Multi-strain bio-fertilizer	>1.0 x 10^9/ml	>0.2 x 10^9/g	>0.1 x 10^9/g

nutrients particularly by their biological activity and help to build up the lost microflora and in turn improve the soil health in general (Ismail et al., 2014). Their mode of action differs and can be used alone or in combination. For easy application, biofertilizers are packed in suitable carrier such as lignite or peat. Carrier also plays an important role in maintaining sufficient shelf life (Singh et al., 1999).

Rhizobium is the most studied and important genera of nitrogen fixing bacteria (Odame, 1997). *Azospirillum* spp. contribute to increased yields of cereal and forage grasses by improving root development in properly colonized roots, increasing the rate of water and mineral uptake from the soil, and by biological nitrogen fixation (Okon, 1985). Biofertilizers have shown great potential as supplementary, renewable and environmental friendly sources of plant nutrients and are an important component of Integrated Nutrient Management (INM) and Integrated Plant Nutrition System (IPNS) (Raghuwanshi, 2012). Naturally grown biofertilizers not only give a better yield, but are also harmless to humans and lead to better sustainable economic development for the farmers and their country (Mishra and Dash, 2014).

ISOLATION TECHNIQUES

Isolation technique for *Rhizobium* spp.

Intact root nodules from a healthy *Sysbania exaltata* plant were selected. One of the pink juvenile root nodule was selected and transferred to a drop of sterile water in a Petri dish. The nodule in the drop of water was crushed in between two glass slides causing the release of nitrogen fixing *Rhizobium* bacteria into the drop of sterile water. The smear of the crushed root nodule was streaked onto yeast extract mannitol agar (YEMA) plate with 1% Congo red dye. The culture was then incubated at 20 to 25°C for three days (Boraste, 2009).

Isolation technique for *Azospirillium* spp.

Juvenile root from a healthy sugar cane plant was taken and kept in saline for 5 min. With a forceps, root was immersed to a semisolid Bromothymol blue medium broth containing 0.8% agar in a test tube and incubated at 20 to 25°C for at least a week. A loopful of culture adjacent to the root in the broth was transferred to bromothymol blue media plates. The culture was incubated at 20 to 25°C for at least a week.

Isolation of phospho bacteria from the rhizoids

1. Soil samples: They are collected from the different agricultural land.
2. Serial dilution method: 10 g of soil sample is dissolved in the 100 ml of distilled water and the sample is mixed well, and by dilution making the sample10^1. Then the soil sample in sterilized water is serially diluted up to 10^7 dilution. Then 10^5, 10^6, 10^7 dilution is taken into spread plate technique.
3. Spread plate technique: Nutrient agar are poured in to the plate, after solidification of medium 0.1 ml of medium are poured into the agar medium plate, then they are incubated at 37°C for 24 h.

Carriers

Carriers (Table 1) increase the effectiveness of the biofertilizer. It enables easy handling and increases the storage or shelf life. Carriers which are used for making solid type of biofertilizer products are clay mineral, diatomaceous soil, and white carbon as mineral; rice, wheat bran, peat, lignite, peat soil, humus, wood charcoal and discarded feed as organic matter. However, clay mineral and rice bran are most often used as carriers. To achieve the tight coating of inoculant on seed surface, use of adhesive, such as gum arabic, methylethylcellulose and vegetable oil is also available.

MASS PRODUCTION OF BIOFERTILIZERS (FIGURE 1)

Criteria for strain selection

Efficient nitrogen fixing strains is selected and then

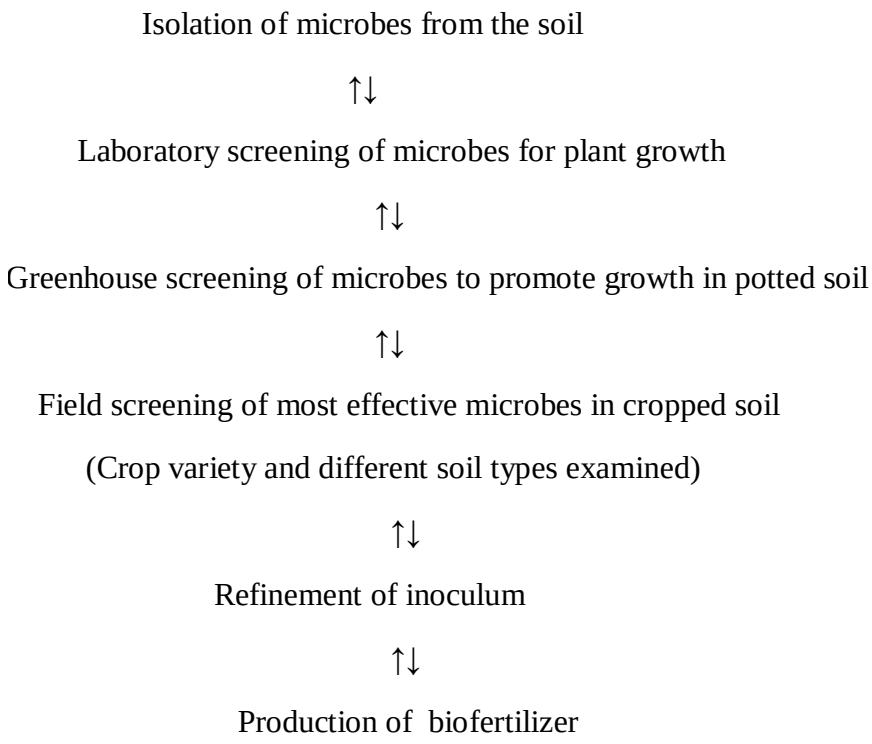

Figure 1. Production of biofertilizers.

multiplied on the nutritutionally rich artificial medium before inoculating in the seed and soil.

Culturing in the flask containing broth

The isolated strain is inoculated in the small flasks containing suitable medium for inoculums production. Now, the carrier was autoclaved at 15 psi at 121°C for 20 min. The culture broth was mixed with the carrier at 30%, that is, for 1 kg carrier; 300 ml of culture broth was used. The mixture was spread on a plastic sheet in a closed room for air drying. The biofertilizer was packed in sterile plastic air tight bags and stored. For large scale production of inoculums, culture fermenters are used.

Quality control

Like every product, the biofertilizers should also follow some standards. The inoculants should be carrier based, and it should contain 10^8 viable cells per gram of carrier on dry mass basis within 15 days of manufacture. The inoculums should have a maximum expiry period of 12 month from the date of manufacture. The inoculants should not have any contaminant. The contaminant is one of the biggest problems faced by the biofertilizers industry. The pH of the inoculant should be 6.0-7.5. Each packet containing the biofertilizer should be marked with the information eg. name of the product, leguminous crop for which intended, name and address of the manufacturer, type of carrier, batch or manufacture no, expiry date. Each packet should also be marked with the ISI mark. The biofertilizer should be stored in the cool place and keep away from direct heat.

Types of biofertilizer (Table 2) available

1. Nitrogen fixing biofertilizer: *Rhizobium*, *Azotobacter*, *Azospirillum*, *Bradyrhizobium*.
2. Phosphorus solubilising biofertilizer (PSB): Bacillus, *Pseudomonas*, *Aspergillus*.
3. Phosphorus mobilizing biofertilizer: *Mycorrhiza* (Plate 1).
4. Plant growth promoting biofertilizer: *Pseudomonas.*

Mode of action of biofertilizer

They fix nitrogen in the soil and the root nodules of the legumes crop and make it available to the plant. They solubilise the insoluble form of the phosphate like tricalcium, iron and aluminium phosphate into the available form. They produce hormones and anti metabolites which promote root growth. They also decompose the organic matter. When biofertilizers are applied to the seed and the soil they increases the

Table 2. Different types of biofertilizers.

Biofertilizer	Microorganism
Nitrogen fixers	*Azolla pinnata, Rhizobium* spp., *Azotobacter chroococcum,* *Azospirillum lipoferum, Acetobacter diazotrophicus, Derxia gummosa*
Phosphate solubilizers	*Bacillus circulans, Bacillus coagulans, Torulospora globasa,* *Pseudomonas fluorescens (siderophore), Thiobacillus (SOM),* *Aspergillus niger (avirulent), Trichoderma* sp., *Paecilomyces sp*
Potash mobilizers	*Bacillus* spp., *Pseudomonas* spp.
Zinc mobilizer	*Pseudomonas* spp., *Bacillus* spp., *Rhizobium* spp.

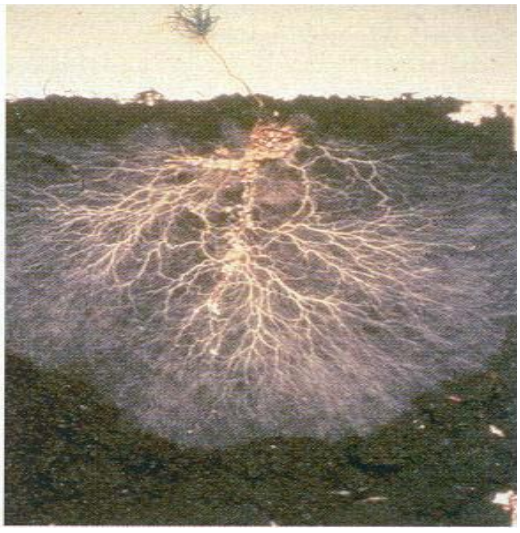

Plate 1. Mycorrhizae (Peters, 2002).

availability of the nutrient to the plant and increases the yield up to 10-20% without producing any adverse effect to the environment. Therefore, significantly increase the plant growth parameters *viz*., plant height, number of branches, number of roots, root length, shoot length, dry matter accumulation in plant organs and vigour index etc. (Ezz El-Din and Hendawy, 2010; Ateia et al., 2009; Mahmoud, 2009; Leithy et al., 2009; Gharib et al., 2008; Ismail et al., 2014).

Phosphorus producing biofertilizer

Phosphate solubilising microorganisms include several bacteria and fungi which can grow in the medium containing tricalcium, iron and aluminium phosphate, hydroxy apatite, bonemeal, rock phosphate and some insoluble phosphate compound. The most efficient PSM belong to the genera *Bacillus* and *Pseudomonas* among bacteria and *Aspergillus* and *Penicillum* amongst fungi (Gaur, 1990). Several varieties of PSM have been isolated from the rhizospheric soil of the crop. Majority of bacterial organisms are known to solubilize phosphate. These bacteria and fungi are used as a biofertilizer. Their application in several crop tend to increase their yield in crop such as cereals, legume, vegetable, fruit crops (Kundu et al., 2009). Phosphate solubilising microorganisms release metabolite such as organic acid latter being converted into the soluble form (Nahas, 1996). Phosphate solubilising microorganisms dissolve soil P through production of low molecular weight organic compound mainly gluconic and ketogluconic acid (Khan et al., 2009).

Low cost medium preparation for PSB

Alternative for King's B broth (*Pseudomonas* spp.); 1. Fish extract, 10 ml; 2, Algal water, 25 ml; 3, *Aloe vera* extract, 5 ml; 4, Tap water, 100 ml; 5, pH, 7.2.

The broth was prepared, inoculated with *P. flourescens* and incubated at 27°C for 24 h on rotary shaker.

Uses of PSB

PSB can be used for all the crops including paddy, millets, oilseeds, pulses and vegetable.

Method of application of PSB

1. Seed treatment: 10 kg of normal size seeds of lentil,

Plate 2. VAMRI.

Plate 3. Brown magic.

mung, berseem treated with 200 g of PSB. Large size seeds like groundnut, chickpea, soyabean require 400-600 g inoculants for 10-12 kg seeds.

2. Seedling dip: This method is useful for the transplanted seedling and also useful for the vegetable crop. Inoculant suspensions are prepared in 1:10 ratio. Dipping the root of the seedlings in this suspension for 5 min.

3. Soil application: 3-5 kg of inoculants is mixed with the 50 kg of farm yard manure (FYM).

Phosphorus deficiency is one of the major limiting factors in crop growth and nitrogen fixation in the tropical regions. Phosphorus requirement is next to the nitrogen. It makes up 0.2% of the body weight. It plays an important role in cell division, cell development, photosynthesis, breakage of sugar, nuclear transport within the plant. Nowadays, phosphorus is a non-renewable and costly input, and these phosphate fertilizers also have pollution problems associated with them. Mycorrhizal fungi (Plate 1) can utilize phosphorus from extremely low concentration. Mycorrhiza based biofertilizer technology is one of such successful technology capable of wasteland reclamation and beneficial in agriculture because it provides phosphorus nutrition to the plant. Mycorrhizae also benefit plants indirectly by enhancing the structure of the soil (Mahdi et al., 2010). AM hyphae excrete gluey, sugar-based compounds called Glomalin, which helps to bind soil particles, and make stable soil aggregates (Peters, 2002).

Marketable product of mycorrhizal fungi

Vesicular Arbuscular Mycorrhiza Root Inoculant (VAMRI) is a chopped dried corn roots infected with arbuscular mycorrhizal fungus, either *Glomus mosseae* or *Glomus fasciculatum*. VAMRI serves as bio-fertilizers and bio-control agents of soil-borne diseases of different crops under various conditions. They show a degree of resistance or tolerance against soil-borne pathogens like nematodes, bacteria and fungi. VAMRI can substantially reduce or substitute the chemical fertilizer and pesticide requirements of crops. This inoculant can be used for pepper, eggplant, tomato, papaya, banana, pineapple, watermelon, onion, corn, sugarcane, peanut, fruit crops/trees and ornamental plants.

Application

VAMRI (Plate 2) can be applied by seed pelleting or coating for direct seeding crops, by mixing with the sowing medium .VAMRI can replace 50-100% of chemical fertilizers.

Brown magic is a mycorrhizal fungal inoculant that can be utilized as biological fertilizer and bio-control agent of root diseases of orchids. This fungus was selected from 200 isolates composed of sclerotium or fruiting bodies of fungi and mycelia collected and isolated from orchid roots.

Application of brown magic (Plate 3)

It increases the growth and survival of *in vitro* cultured orchid and it also increases the tolerance and resistance of plants to pathogens and diseases; induces early flowering and enhances the production of more suckers and longer spikes. This inoculant is environment-friendly, economical and easy to use. This inoculant is available at BIOTECH Sales office at UPLB.

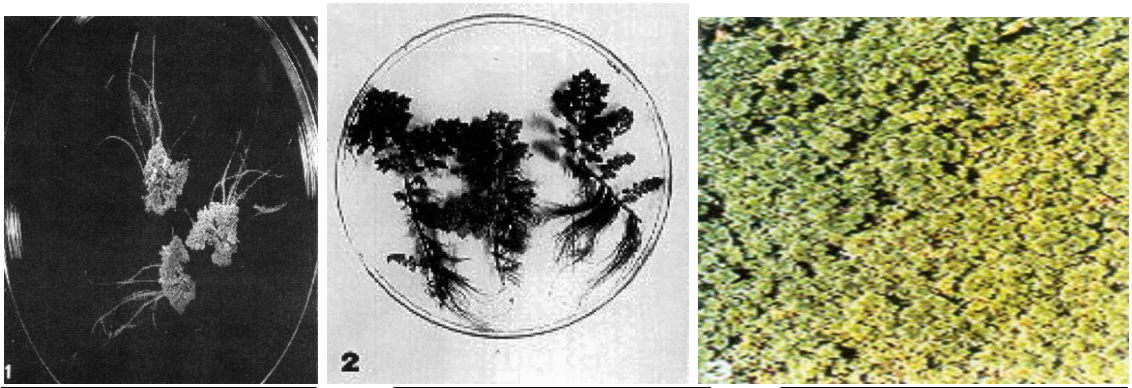

Plate 4. Different types of *Azolla* spp. (Pabby et al., 2004).

Application of mycorrhizae

Endomycorrhizae should be applied at a rate of 3,600,000 propagules per Mycorrhizae via hand seeding, seed drilling, hydroseeding, broadcast and till, planting, or as a nursery medium.

Facultative endophytic diazotroph (*Azospirillum*)

Azospirillum belongs to family Spirilaceae. It is heterotrophic and associative in nature. It has also an ability to fix nitrogen of about 20-40 kg/ha, they also produce growth regulating substances. The different species of *Azospirillum* are *Azospirillum amazonense, Azospirillum halopraeferens, Azospirillum brasilense*. The *Azospirillum* form associative symbiosis with many plants particularly with those having the C4-dicarboxyliac path way of photosynthesis (Hatch and Slack pathway), because they grow and fix nitrogen on salts of organic acids such as malic, aspartic acid (Arun, 2007). It is mainly used for some recommended crops like maize, sugarcane, sorghum, pearl millet etc. *Azospirillum* species belong to the carbon compounds and adequately low level of facultative endophytic diazotrophs groups which colonize combined nitrogen (Andrew et al., 2007). *Azospirillum*, directly benefits plants by improving the fixing activity of bacteria in rhizosphere of plants (Nghia and Gyurjan, 1987). It helps in shoot and root development (Gonzalez et al., 2005).

Obligate endophytic diazotrophs (*Azotobacter*)

Azotobacter is an obligate aerobe, bacteria of the genus *Azospirillum* are a well-known or endocytobionts live in special cells of their hosts which are known as *Rhizobia* found in the root nodules of legumes or *Frankia* widespread in the soils of tropical, subtropical. Azotobacter as nitrogen-biofertilizer increase the growth and yield of various crops under field conditions (Table 3). These bacteria develop in close-legume symbiosis which is one of the most efficient fixing relationships with the roots of various wild plants (Doroshenko and Rawia, 2007; Rawia et al., 2009).

Algal biofertilizers (N fixers)

***Azolla* spp.**

It is a diazotrophic symbiont, it is well known for its utilization as a nitrogen fertilizer. *Azolla* spp. (Plate 4) float in water in large number and in soils where there is appropriate number of fern species. These are mostly found in the tropical and temperate ecosystems. There is a symbiotic association between *Azolla* and a *cyanobacteria Anabaena*. The host *Azolla* provide carbon source to the anabaena while its nitrogen requirement is fulfil by atmospheric nitrogen fixation by *Cyanobacteria*. It has the ability to fix atmospheric nitrogen through bacteria and the infection always occurs during the life of symbiosis with blue green algae (*Nostoc anabaena*).They are the potential source of nitrogen especially for wet land rice (Table 4). The contribution of nitrogen from *Azolla* spp. to wet land rice has been found to be maximum when incorporated into the soil as green manure (Galal, 1997). The benefit of growing azolla as a biofertilizer for both N and K is its usefulness as human feed and it is also used as a mosquito repellent. An increase in the yield of paddy ranging from 9-39% has been obtained in the field experiment when *Azolla* was incorporated in the soil (Singh, 1977).

Cyanobacteria

N_2 fixing *Cyanobacteria* are most wide spread N_2 fixers on earth. *Cyanobacteria* or blue green algae are the

Table 3. Effect of *Azotobacter* on crop yield.

Crop	Increase in yield over yields obtained with chemical fertilizers (%)	Crop	Increase in yield over yield obtained with chemical fertilizers (%)
Food grains		Other	
Wheat	8-10	Potato	13
Rice	5	Carrot	16
Maize	15-20	Cauliflower	40
Sorghum	15-20	Tomato	2-24
		Cotton	7-27
		Sugarcane	9-24

Table 4. *Azolla* spp. which can be used as biofertilizer.

***Azolla* spp.**	**Reference**
A. pinnata	Singh and Srivastava, 1984
A. mexicana	Thanh and Hang, 1988
A. filiculoides	Singh and Srivastava, 1984
A. rubra	Stergianou and Fowler, 1990
A. niloticaa	Stergianou and Fowler, 1990
A. caroliniana	Thanh and Hand, 1988
A .microphylla	Stergianou and Fowler, 1990

diverse group of prokaryotes. The activities of nitrogen-fixing organisms provide an important source of nitrogen to the marine eco system (Gonzalez et al., 2005). They also grow and fix nitrogen in terrestrial environment, from rain forest to desert (Peter et al., 2002). *Cyanobacteria* are able to survive in the extreme environment and have ability to fix nitrogen because of the capacity to fix nitrogen; they are used as a bio fertilizer. In addition to contributing N, the *Cyanobacteria* add organic matter, secrete growth promoting substance like auxin, vitamins, mobilise insoluble phosphate and improve physical and chemical nature of the soil. *Cyanobacteria* act as a supplement to the N fertilizers contributing up to 30 kg N/ ha. It increases the crop yield between 5-25%.

Mass multiplication of *Azolla*

For mass multiplication of *Azolla*, microplots (20 m^2) are prepared in the nurseries in which sufficient water (5-10 cm) is added. For profuse growth of *Azolla* 4-20 kg P_2O_5 is amended. Optimum pH 8.0 and temperature of 14-30°C should be maintained. Finally, microplots are inoculated with fresh *Azolla*. An insecticide (Furadon) is used to check the insect's attack. After 3 weeks, the mat of *Azolla* is ready for harvest and the same microplots are inoculated with fresh azolla to repeat the cultivation. Azolla mat is harvested and dried to use as green manure.

Mass multiplication of *Cyanobacteria*

The following methods are used for mass cultivation:

a) Cemented tank method
b) Shallow metal trough method
c) Polythene lined pit method
d) Field method

i) Prepare the cemented tank, shallow trays of iron sheets are polythene lined pits in an open area. Width of tanks or pits should not be more than 1.5 m.This will facilitate the proper handling of culture.
ii) Transfer 2-3 kg soil and add 100 g superphosphate. Water the pit to about 10 cm height, Mix lime to adjust the pH. Add 2 ml of insecticides to protect the culture from mosquitoes. Mix well and allow to settle down soil particles.
iii) When water become clear, sprinkle 100 g starter culture on the surface of water.
iv) When temperature remains around 35-40°C during summer, optimum growth of *Cyanobacteria* is achieved. The water level is always maintained at about 10 cm during the period.
v) After drying, the algal mass is separated from the soil that form flaskes. Then it is collected, powdered and packed in the polythene bag and supplied to the farmers after sealing the packets.
vi) The algal flakes can be used as starter inoculums again.

Gluconacetobacter diazotrophicus

G. diazotrophicus is a nitrogen-fixing, acetic acid bacterium first isolated from sugarcane plants. It belongs to phylum *Proteobacteria* (comprising Gram negative bacteria) section a-Proteobacteria, order *Rhodospirillales* and family *Acetobacteraceae*. Currently, this family contains three nitrogen-fixing genera, comprising of seven species, namely *Acetobacter nitrogenifigens, Gluconacetobacter kombuchae, Gluconacetobacter johannae Gluconacetobacter azotocaptans, G.*

Table 5. Biofertilizers which are used against crops.

Biofertilizer	Recommended crop	Fertilizer saving
Azolla pinnata (fresh)	Low land rice	30-50 kg N
Azolla pinnata (dry)	Wheat, potato, tobacco	30-50 kg N
BGA	Low land rice	30-50 kg N
Azotobacter chroococcum	Pearlmillet,sorghum, rajma, sugarcane, maize, potato, pigeonpea, onion, cotton	20-40 kg N
Azospirillum lipoferum	Pearlmillet, fingermillet, paddy, sorghum, maize, tobacco, onion	20-40 kg N
Acetobacter diazotrophicus	Sugarcane	100 kg N
Rhizobium spp	Pigeonpea, chickpea, greengram	30-50 kg N
Bacillus circulans	Cow pea	20-50 kg P_20_5
Bacillus brevis	Sorghum, wheat, pearlmillet	20-50 kg P_20_5
Bacillus congulans	Sorghum, cowpea, pearlmillet, groundnut	20-50 kg P_20_5

diazotrophicus, Swaminathania salitolerans and *Acetobacter peroxydans*.

Rhizobium

Rhizobium belongs to family *Rhizobiaceae,* it is symbiotic in nature, it fixes 50-100 kg/ha nitrogen with legumes only. It includes the following genera: *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium* and *Allorhizobium* (Vance, 2001; Graham and Vance, 2000). It is useful for the pulse legumes like chickpea, red-gram, pea, lentil, black gram, etc., oil-seed legumes like soybean and groundnut and forage legumes like berseem and lucerne (Table 5). It colonizes the roots of specific legumes to form tumour like growths called root nodules, which acts as factories of ammonia production.

Rhizobium has ability to fix atmospheric nitrogen in symbiotic association with legumes and certain non-legumes like *Parasponia*. Population of the *Rhizobium* population in the soil depends on the presence of legume crops in the field. In the absence of legumes, the population decreases.

Frankia (N fixers)

Frankia is the genus of N2-fixing actinomycetes (Benson and Silvester, 1993; Huss-Danell, 1997). These are also called actinorhizal plants and they are also used in land reclamation, for timber and fuel wood production, in mixed plantations, for windbreaks, (Schwencke and Carù, 2001). *Frankia* N_2 fixation has been estimated to be similar to rhizobial symbioses (Torrey, 1978; Dawson, 1986; Dommergues, 1995).

Plant growth promoting rhizobacteria

Various bacteria can promote plant growth (Bashan, 1998). Collectively, such bacteria are called plant-growth-promoting rhizobacteria (PGPR). These bacteria vary in their mechanism of plant growth promotion but generally influence growth via P solubilization, nutrient uptake enhancement, or plant growth hormone production (Bashan et al., 1990; Okon and Labandera-Gonzalez, 1994; Goldstein et al., 1999; Richardson, 2001). Bertrand et al. (2000) showed that a rhizobacterium belonging to the genus *Achromobacter* could enhance root hair number and length in oilseed rape *(Brassica napus*).

POTENTIAL ROLE OF BIOFERTILIZER IN AGRICULTURE

The biofertilizers play an important role in improving the fertility of the soil (Kachroo and Razdan, 2006; Son et al., 2007). In addition, their application in soil improves the structure of the soil minimizes the sole use of chemical fertilizers. Under low land conditions, the application of BGA + *Azospirillum* proved significantly beneficial in improving LAI. Grain yield and harvest index also increase with use of biofertilizers. Inoculation with *Azotobacter* + *Rhizobium* + VAM gave the highest increase in straw and grain yield of wheat plants with rock phosphate as a P fertilizer. *Azolla* is inexpensive, economical, friendly, which provide benefit in terms of carbon and nitrogen enrichment of soil (Kaushik and Prassana, 1989). Some commercially available biofertilizers are also used for the crop (Table 6). Raj (2007) recorded that microorganisms (*B. subtilis, Thiobacillus thioxidans* and *Saccharomyces* sp.) can be used as bio-fertilizers for solubilization of fixed micronutrients like zinc. Soybean plants, like many other legumes can fix atmospheric nitrogen symbiotically and about 80 to 90% nitrogen demand could be supplied by soybean through symbiosis (Bieranvand et al., 2003). Bio-control, a modern approach of disease management can play a significant role in agriculture (Tverdyukev et al., 1994; Hoffmann-Hergarten et al., 1998; Yang-Xiu Juan et al*.,* 2000; Sharon et al., 2001; Senthilkumar and

Table 6. Commercially available biofertilizer and their manufacture, beneficial crop and associated microorganisms.

Product	Manufacture's name	Microbe used	Benefical crop
Nitragin TM	Nitragin Sales Corpn.Wisconsin, 53209	Rhizobium	Soyabean
Rhizocote	Coated Seed Ltd, Nelson, New Zealand	Rhizobium	Legumes
Nodosit	Uniob Chemiques S.A. Belgium	Rhizobium	Legumes
Rhizonit	Phlylaxia Allami Budapest, Hungary	Rhizobium	Legumes
Nitrazina	Wytwornia Walcz Poland	Azotobacter	Cereals and vegetables
N-germ	Laboratoire de Microbiologie, France	BGA	Rice
Tropical inoculants	Tropical inoculants	Azotobacter	Rice and wheat
Nodulaid	Brisbane, Queensland Agricultural Lab. New South Wales, UK	Rhizobium	Legume
Azotobacterin	Tashkent laboratories Moscow	Azotobacter	Vegetable and cereals.
Nodion	Indian Organic Chems. Ltd. Mahew Mahal, Bombay	Rhizobium	Legumes
Azoteeka	Bacifil, 25 Nawal Kishore Rd.Lucknow	Azotobacter	Cereals
Agro-teeka	National Fertilizers and Chemicals 11, Ind Area-II, Ramdarbar, Chandigarh	Azotobacter	Wheat, rice, maize, tea, sugarcane, potato.
Rhizoteeka	Microbes India,87.Lenin Savabe, Calcutta	Rhizobium	Legumes
Nitrogeron	Root Nodne Pvt. Ltd. Australia.	Rhizobium	Legumes

Rajendran, 2004; Li-Bin et al., 2005; Hossain et al., 2009). *Trichoderma* based BAU-biofungicide has been found promising to control root knot diseases of French bean (Rahman, 2005). Use of antagonist bacteria like *Rhizobium* and *Bradyrhizobium* also has significant effect in controlling root knot of mungbean (Khan et al., 2006). Growth, yield and quality parameters of certain plants significantly increased with biofertilizers containing bacterial nitrogen fixer, phosphate and potassium solubilizing bacteria and microbial strains of some bacteria (Youssef and Eissa, 2014).

Constraints in the use of the biofertilizer

1. Unavailability of suitable strain: Due to the lack of the availability specific strain it is one of the major constraint in the production of the biofertilizer. Based on the fact that selective strain have ability to survive both in the broth and the inoculants carrier.
2. Unavailability of suitable carrier: If suitable carrier is not available it is difficult to maintain the shelf life of the biofertilizer. As per the suitability, the order is peat, lignite, charcoal, FYM, soil, rice husk. Peat of good quality is rarely found in India. Good quality carriers have a good moisture holding capacity, free from toxic substances.
3. Lack of awareness among farmers: Farmers of India are not aware of the biofertilizers, their usefulness in increasing crop yields.
4. Inadequate and inexperienced staff: This is because the unskilled and the inadequate staff farmers are not given proper instruction about the application. The production of biofertilizers in the country is 10,000 mt/annum and the production capacity is 18,000 mt/annum. Average annual consumption of biofertilizers in the country is about 64 g/ha.

Limitation of biofertilizer

1. Biofertilizers never mix with the chemical fertilizers.
2. Biofertilizers are never applied with the fungicides, plant ash at a same time.
3. Biofertilizers are never exposed to direct sunlight.
4. Stored at room temperature not below 0 and 35°C.

CONCLUSION

Biofertilizers are becoming increasingly popular in many countries and for many crops. Biofertilizers are fertilizers containing living microorganisms, which increase microbial activity in the soil. Often, organic food is included to help the microbes get established. In India soil fertility is diminishing gradually due to soil erosions, loss of nutrition, accumulation of toxic elements, water logging and unbalanced nutrient compensation. Organic manure and bio fertilizers are the alternate sources to meet the nutrient requirement of crops. biofertilizers, benefiting the crops are *Azotobacter, Azosprillium, Phosphobacter* and *Rhizobacter* which are very important. The role of biofertilizer in agricultural production is of great importance. Inoculation of nitrogen fixing bacteria with biofertilizer increases the phosphorus level that influences the sunflower seed oil content and the proportion of fatty acids

(unsaturated/saturated fatty acids ratio). Biofertilizers can also make plant resistant to adverse environmental stresses. Control of root-knot disease of soybean caused by *Meloidogyne javanica* may be explored through use of BAU-Biofungicide and BINA-Biofertilizer for eco-friendly management of this nemic disease avoiding chemical nematicides. The proper application and use of biofertilizers will not only have an impact on sustainable agriculture's economic development but it will also contribute to a sustainable ecosystem and the holistic well-being.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Seroprevalence of contagious caprine pleuropneumonia and field performance of inactivated vaccine in Borana pastoral area, southern Ethiopia

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This study was conducted between September 2012 and May 2013 in three Districts of Borana pastoral area to determine seroprevalence of contagious caprine pleuropneumonia (CCPP) and assess field performance of inactivated commercial vaccine, produced by the National Veterinary Institute (NVI) in Ethiopia, against CCPP. Both pre and post vaccination sera samples were tested using competitive enzyme linked immunosorbent assay (cELISA). Out of 510 examined sera, 161 samples were positive for CCPP, giving an overall seroprevalence of 31.6% (95% CI = 27.57-35.64%) in the study area. Seroprevalence of 35.2, 35.1 and 25% were recorded in Arero, Dhas and Yabello districts, respectively. However, there was no significant difference ($\chi 2=5.56$, P=0.062) in seropositivity among the three districts and between male and female goats ($\chi 2=0.068$, P=0.794) examined in this study. On the other hand, the differences in seroprevalence among the age categories were statistically significant ($\chi 2=24.48$, p<0.0001). A rise in antibody (seroconversion) was observed after field vaccination of goats with inactivated CCPP vaccine and a total of 253 of the 414 examined sera samples were positive for *Mycoplasma capricolum* subsp. capripneumoniae (*Mccp*) specific antibodies, thus 61.1% of goats seroconverted following vaccination. Comparison of *Mccp* specific antibodies in the goat population before and after vaccination indicated that the number of positive reactors increased significantly (P<0.0001) following CCPP vaccination. Seropositivity following vaccination was analyzed among the three age groups and statistically significant differences ($\chi 2=45.48$, P<0.0001) were recorded. The change in the serum antibody after vaccination was found to be higher in younger and adult aged goats than old aged goats. In conclusion, the present study indicates that CCPP is one of the major goat health problems in Borana pastoral area which warrants appropriate measures to be in place towards the prevention and control of the disease. Moreover, field vaccination of goats by inactivated CCPP vaccine induced seropositivity in majority of the inoculated goats. Future controlled experimental studies with challenge infection after vaccination need to be conducted for further evaluation of the vaccine efficacy.

Key words: Borana pastoral area, contagious caprine pleuropneumonia (CCPP), goats, inactivated vaccine, seroconversion, seroprevalence.

INTRODUCTION

Ethiopia possesses an estimated 22.6 million heads of goats (CSA, 2012). Although goats represent a great national resource, their productivity is sub-optimal. Among the several factors that hamper the productivity of this animal, diseases take a lion share. Contagious caprine pleuropneumonia (CCPP) is one of the most important infectious diseases of goats that pose a significant threat to production capacities of this animal. It is a highly contagious and severe respiratory disease caused by *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*) (OIE, 2008).

The presence of CCPP in Ethiopia had been suspected since 1983 and was confirmed later in 1990 by isolation and identification of *Mccp* following an outbreak of CCPP in Ogaden, Eastern Ethiopia (Thiaucourt et al., 1992). Since then the disease has been known to be endemic in different regions of the country (Sharew et al., 2005). Outbreaks of CCPP have been reported from almost all regions of the country, especially from the lowland areas, which are known goat-rearing regions (APHRD, 2010). The frequently reported outbreaks of CCPP in Ethiopia almost certainly represent an underestimate as this disease is having a major socio-economic impact in the country (Nicholas and Churchward, 2012).

Borana rangeland area is among the known pastoral areas of Ethiopia and possesses huge livestock resource. Goats being an important component of livestock play a significant role in supporting the pastoralist's livelihood in the area (CARE-Ethiopia, 2009). Despite the high population density of goats estimated at 849,261 (CSA, 2012) in the Borana pastoral area, little attention has been given to the health problems of goats. To date there has not been sufficient study on CCPP in the area; hence there is dearth of well-documented information on the current status and precise distribution of the disease in the area.

CCPP is a highly contagious disease; hence control of the disease is one of the priority areas of the country. Like most African nations, vaccination remains the most cost effective strategy to control animal disease in Ethiopia. To realize success in vaccination campaigns, use of effective vaccine is crucial (AU-IBAR, 2013). From past pilot experiments conducted in Kenya, inactivated CCPP vaccines consisting of saponized organisms have been shown to be protective but the quality and efficacy may be variable (Rurangirwa et al., 1987a, b). Vaccine against CCPP is currently produced by the National Veterinary Institute (NVI) in Ethiopia, which is inactivated *Mccp* vaccine with saponin as an adjuvant. This vaccine is extensively used for the control of the disease in endemic areas of the country (APHRD, 2010). Experimental trials conducted in the country have demonstrated that immunization of goats with inactivated *Mccp* (F-38 Kenyan strain) in adjuvant confers protection against a contact challenge (Ayelet et al., 2007). However, the efficacy of inactivated *Mccp* vaccine under field conditions has not been clearly documented.

The current study was conducted in Borana pastoral area, the largest pastoral area in Ethiopia, to determine the seroprevalence of CCPP and assess field performance of the currently available inactivated vaccine against the disease, regarding its seroconversion level following vaccination of goats in Borana pastoral area.

MATERIALS AND METHODS

Description of the study area

The study was conducted between September 2012 and May 2013 in three districts (Yabello, Arero and Dhas) of Borana pastoral area, Southern range lands of Ethiopia. Borana zone is under Oromia regional state and comprises of mainly pastoral areas and seldom agro-pastoral areas. Yabello, Arero and Dhas districts are among the pastoral areas and located at distance of 570 to 665 km from Addis Ababa, the capital city of Ethiopia.

Study animals

The study animals were local Borana goat breeds (Long-eared Somali breeds) managed under extensive pastoral production system by the Borana pastoralists (Gizaw, 2009). Goats with no history of vaccination for CCPP and above 6 months of age were used as source of sera samples for the study. Goats of both sex and various age groups were sampled. Age of the animals was determined based on owners information and dental eruption; accordingly, animals were categorized into three age groups: > 6 months and ≤ 2 years (young), >2 years and ≤5 years (adult) and >5 years (old) (Bekele et al., 2011).

Study design

A cross-sectional survey was carried out on goats in three districts of Borana Zone to determine seroprevalence of CCPP and assess post-vaccinal antibody response of inactivated CCPP vaccine at field level. Blood samples were collected twice before vaccination and three weeks post vaccination from jugular vein of individual goats. Sera samples were tested using competitive enzyme linked immunosorbent assay (cELISA) according to the standard test procedure at the National Veterinary Institute (NVI) of Ethiopia.

Sample size and sampling method

The three study districts (Yabello, Arero and Dhas) were selected purposively. Pastoral associations and villages (locally known as

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Olla) under each district were considered as primary and secondary sampling units used for random sampling, respectively. Pastoral associations (PAs) were selected randomly from each district after obtaining the total number of PAs in the districts. Subsequently 3 PAs were selected from each district and a total of 9 PAs (one village from each PA) were considered for the present study. The total number of samples in each district and PAs were allocated proportionally.

Systematic random sampling method was employed for sampling of individual animals. Animals below six months of age were excluded from sampling. Animals were selected systematically the first animal being selected randomly from the flock then at an interval until the required number of sample size in that village was attained; this was done early in the morning before animals were released for grazing. During sampling, history of goats whether they were the member of the flock or not were asked and a newly recruited goats replaced by existing flocks to avoid the risk of including vaccinated animals.

The sample size was calculated according to the formula given by Thrusfield (2007) by considering 20% expected prevalence in the area (Gelagay et al., 2007), using 95% confidence interval at 5% absolute precision. Thus, the calculated sample size was 246. In order to increase precision, the sample size was doubled to get a total of 510 pre-vaccination blood samples. For the field assessment of inactivated CCPP vaccine, it was possible to collect post-vaccination blood samples from 414 previously sampled (pre-vaccinal blood sample) and vaccinated goats. However, from previously sampled animals, 96 goats were missed during the second round sample collection (post-vaccinal sample collection).

Study methodology

Blood sample collection

Blood samples were collected twice from individual goats, that is before vaccination and three weeks post vaccination. The separated sera were stored in deep freezer at -20°C at Yabello Regional Veterinary Laboratory until processed at the NVI serology laboratory. Transportation to the National Veterinary Institute (NVI) laboratory was done by placing packed samples in an ice box containing preformed blocks of ice.

Vaccination

Inactivated CCPP vaccine, commercially produced by NVI (Ethiopia) from F-38 Kenyan strain of *Mccp,* was used to vaccinate goats in Borana pastoral area. After shaking gently, 1 ml/goat was injected subcutaneously under the loose skin at the neck region using automatic vaccination syringe. To follow rise in antibody (seroconversion level), 5-7 ml of blood sample was collected 3 weeks post-vaccination (at 21^{th} day). Due to difficult working conditions in the pastoral area, it was not possible to follow up any post vaccination reactions and repetitive collection of blood samples from individual goats after vaccination was not possible too.

Serological test

Collected sera samples were examined for the presence of specific antibodies against *Mccp* by using cELISA in serology laboratory of the National Veterinary Institute (Ethiopia). The cELISA test was employed using *Mccp* antibody test kit and it was obtained from CIRAD-Montpellier, France. Similar studies have used cELISA kit to detect antibodies that appear after an infection or after an immunization with a relevant CCPP vaccine (Peyraud et al., 2014). Thus, the same kit was used to determine the disease prevalence and to measure antibodies following vaccination.

The 924 test sera (both pre and post vaccination sera samples) were examined in twelve 96-well flat bottom microplates, according to the test protocol supplied with the kit. Test samples and controls were pre-diluted on the preplate (uncoated). Samples to be tested were premixed with a specific monoclonal anti-*Mccp* antibody (Mab 4.52) in a preplate (uncoated) and homogenized contents of the preplate were transferred into the *Mccp* antigen coated microplate. The contents were incubated for 1 h at 37°C with a gentle agitation, washed two times, dried and then an anti-mouse IgG enzyme conjugate was dispensed and incubated for 30 min. Then, after three times washing enzyme substrate was added to be incubated for 20 min. Finally, stop solution was added and color development was observed and read at 450 nm by ELISA reader to determine the optical density and percentage of inhibition was calculated. For the assay to be valid, results of internal quality control sera were first checked to make sure they are within the acceptable ranges. Those samples with percentage of inhibition greater than or equal to 50% are considered positive for presence of *Mccp* antibodies.

Data analysis

All collected data were entered into Microsoft Office Excel 2007 computer program and then summarized first by using a descriptive statistics. All statistical analyses were performed using Statistical Package for Social Science (SPSS)-Version 20. Seroprevalence of CCPP and post-vaccination seroconversion percent was calculated as the proportion of the number of cELISA positive animals to the total number of tested animals expressed in percent. Chi-square test was used to assess association of the disease with districts, age group and sex. Paired-sample t-test was used to analyse if there is difference between pre and post vaccination antibody titer (paired scores). A P-value less than 0.05 at 95% confidence interval was considered for significance.

RESULTS

Seroprevalence

Out of 510 examined sera, 161 samples were positive for *Mccp* specific antibodies using cELISA. An overall seroprevalence of 31.6% (95% CI= 27.57-35.64%) was observed in the study area (Table 1). The seroprevalence was highest in Arero district (35.2%) followed by Dhas (35.1%) and the lowest seroprevalence was recorded in Yabello (25%). There was no significant difference (χ^2=5.56, P=0.06) in CCPP seroprevalence among the three districts. Analysis of the seroprevalence of CCPP with respect to sex showed no significant difference (χ^2=0.068, P=0.79) between male and female goats (Table 2).

The result of age groups were compared for seropositivity of CCPP as indicated in Table 3. Seroprevalence of 26.4, 25.8 and 50.4% were recorded in young, adult age and old age categories, respectively.

The differences in seroprevalence among the age categories were significant (χ^2=24.48, p<0.0001). Old age category has showed significantly higher seroprevalence as compared to the young and adult age category. The odds of the disease in old age category was 2.83 (95%

Table 1. Seroprevalence of CCPP in the three districts of Borana pastoral area, Ethiopia.

District	Sample tested	Sample positive	Seroprevalence (%)	95% Confidence interval
Yabello	180	45	25	18.67-31.33
Dhas	168	59	35.1	27.88-42.32
Arero	162	57	35.2	27.85-42.55
Total	510	161	31.6	27.57-35.64

Table 2. Seroprevalence of CCPP with respect to sex in Borana pastoral area, Ethiopia.

Sex	Sample tested	Sample positive	Seroprevalence (%)	95% Confidence Interval	OR (95% CI)
Male	172	53	30.8	23.9-37.7	1
Female	338	108	32	27.03-36.97	1.05 (0.71-1.57)
Total	510	161	31.6	27.57-35.64	

Table 3. Seroprevalence of CCPP in different age groups in Borana pastoral area, Ethiopia.

Age	Sample tested	Sample positive	Seroprevalence (%) (95% CI)	P-value	OR (95% CI)
Adult	221	57	25.8 (20.03-31.57)		1
Young	174	46	26.4 (19.85-32.95)	0.885	1.03 (0.658-1.625)
Old	115	58	50.4 (41.26-59.54)	0.000	2.93 (1.82-4.70)
Total	510	161	31.6 (27.57-35.64)		

CI=1.72-4.65) and 2.93 (95% CI=1.82-4.70) times to occur as compared to the young and adult age categories, respectively.

Post vaccination seroconversion

A rise in antibody (seroconversion level) was assessed after vaccination of goats with inactivated CCPP vaccine. Paired sera samples (pre- and post-vaccination) were collected from 414 vaccinated goats. The serum antibody level was monitored three weeks (at 21th day) post vaccination; thus, out of 414 examined serum samples, 253 were positive for *Mccp* specific antibodies after vaccination using cELISA.

Thus seroconversion was observed in 61.1% of the vaccinated goats of various age groups and both sexes in the three districts of Borena zone (Table 4). Comparison of *Mccp* specific antibody levels in the goat population before and after vaccination indicated that the number of positive reactors increased significantly (P<0.0001) following CCPP vaccination. Moreover, paired sample t-test analysis indicated that there was statistically significant difference between pre and post vaccination antibody level based on the mean percentage of inhibition (t= 9.82, P<0.0001) (Table 5).

When considering only those goats which were negative for *Mccp* specific antibodies in the pre-vaccination sera, a total of 132 (48.4%) of the 273 examined sera samples were positive after vaccination. However, sera samples collected from 141 (51.6%) vaccinated goats remained seronegative for *Mccp* specific antibodies. Seropositivity following vaccination was analyzed among the three age groups and statistically significant differences (χ^2=45.48, P<0.0001) were recorded (Table 6). The change in the serum antibody after vaccination was found to be higher in younger goats (61.45%) followed by adult age goats (56.6%) and the lowest seropositivity following vaccination were recorded among old age goats (8.8%). Analysis of serological response following vaccination among goats in the three districts as well as between sexes showed no significant differences.

DISCUSSION

In Ethiopia, goat rearing carries tremendous importance in house hold economy, especially in pastoral areas where goats are raised in large number (Hirpa and Abebe, 2008). CCPP is one of the most important infectious diseases of goats that pose a significant threat

Table 4. *Mccp* specific antibody levels in the goat population before and after vaccination.

Seroconversion level by	Category	No. of goats from which paired sera samples collected	No. of seropositive goats pre-vaccination (%)	No. of seropositive goats post-vaccination (%)
District	Yabello	163	43 (26.4)	107 (65.6)
	Dhas	120	43 (35.8)	71 (59.2)
	Arero	131	55 (42)	75 (57.3)
Sex	Female	264	94 (35.6)	166 (62.9)
	Male	150	47 (31.3)	87 (58)
Age	Young	139	43 (30.9)	94 (67.6)
	Adult	167	47 (28.1)	115 (68.9)
	Old	108	51 (47.2)	44 (40.7)
Overall		414	141 (34.1)	253 (61.1)

Table 5. Comparison of pre and post vaccination antibody level based on mean percentage of inhibition.

Antibody level during	Sample tested	Mean percentage of inhibition	Paired differences	t-score	P-value
Pre vaccination	414	46.54			
Post vaccination	414	57.2	10.67	9.82	0.000

Table 6. Seroconversion following field vaccination among goats that were negative for *Mccp* specific antibodies before vaccination.

Seroconversion level among age groups	No. of seronegative goats for *Mccp* specific antibodies pre-vaccination	No. of seropositive goats post vaccination	Seroconversion (%)	P-value
Young	96	59	61.45	0.000
Adult	120	68	56.66	0.000
Old	57	5	8.8	
Overall	273	132	48.4	

to production capacities of this animal. The first objective of the present study was to determine the prevalence of contagious caprine pleuropneumonia in Borana pastoral area.

The overall seroprevalence of CCPP in the study areas was 31.6% (95% CI= 27.57-35.64%). The finding in the present study was in line with reports of Hadush et al. (2009) 32.68% in Tigray and Afar, Sharew et al. (2005) 29% in Wollo and Eshetu et al. (2007) 31% in an export abattoir from goats that had been collected from Borana, Afar, Bale and Jinka. Similarly, Sherif et al. (2012), Mohammed (2008) and Ingle et al. (2008) had reported seroprevalence of 32.63% in selected districts of Jijiga Zone, 32% in Eastern Ethiopia and 33.67% in Nagpur District of Vidarbha region of India, respectively.

The overall prevalence of CCPP in the present study was higher than some of previous studies which were conducted in Southern part of Ethiopia. A relatively lower seroprevalence of 18.61% in South Omo and Arbaminch areas (Mekuria and Asmare, 2010), 15.5% in Hammer and Benna-Tsemay (Mekuria et al., 2008), 20.12% in Borana pastoral area (Gelagay et al., 2007) and 13.2% in Borana and Guji lowlands (Bekele et al., 2011) had been reported. Moreover, Eshete (2006) had also reported seroprevalence of 19.19% using cELISA in Afar pastoral area. However, the prevalence recorded in this study was lower than that of Sharew et al. (2005) who reported prevalence rates ranging from 52 to 100% using CFT and B-ELISA in outbreak samples from the lowland districts of the country.

The variation in the seroprevalence of CCPP reported from different studies may be as a result of the temporal and spatial factors associated with sampling, the situation of the disease during the time of sampling and the variation in the specificity and sensitivity of the different serological tests employed.

The difference in seroprevalence between the three districts was not statistically significant (P=0.062). This

may be associated with the non restricted animal movement between this neighboring districts and the highly contagious nature of *Mccp* infection, as well as similar climatic factors in the districts. Since pastoralism is the mainstay of livelihood in the study districts, there has been regular mixing of flocks at watering points and communal grazing areas, which is likely to spread the infection between flocks. This may be explained by the fact that the infection needs proximity to source of infection (Thiaucourt and Bolske, 1996).

Sex of the animal was not associated with seropositivity in this observation. This result was agreeable with the observations made by Eshete (2006), Mekuria and Asmare (2010), Bekele et al. (2011), Yousuf et al. (2012) and Sherif et al. (2012) in studies conducted in different parts of Ethiopia. It has also been reported that CCPP is highly contagious and fatal disease affecting susceptible goats of both sexes (OIE, 2008).

Old age category has showed significantly higher sero-prevalence as compared to the young and adult age category ($p<0.0001$). Similarly, significant variation among age groups was also reported by Mekuria and Asmare (2010), Regassa et al. (2010), Yousuf et al. (2012) and Sherif et al. (2012). In all these studies, it was reported that the prevalence of the disease was significantly higher in old age than young animals. The higher prevalence of the disease in old age goats as compared to the young and adult ages might be explained by the fact that as age increases, the goats are often repeatedly exposed to different stress conditions (due to malnutrition, movement over long distances, adverse weather conditions and the likes) which can predispose the animal to the disease. Advancing age in goats, as with other species, is eventually associated with a decline in body condition as well as an increase in susceptibility to infections. Moreover, they also tend to be infected repeatedly. Therefore, the probability to be seropositive in older ages for CCPP would be high as compared to young and adult goats. However, there was also a report that suggested absence of age factor in CCPP epidemiology (Eshetu et al., 2007; Hadush et al., 2009). Perhaps this assumption needs further investigation.

With the next objective of the present study, some observations were done on field performance of the inactivated CCPP vaccine after vaccination of goats in Borana pastoral area. A rise in antibody was assessed and seroconversion was observed in 61.1% of the vaccinated goats. Comparison of *Mccp* specific antibodies in the goat population before and after vaccination indicated that the number of positive reactors increased significantly following CCPP vaccination. Thus, the inactivated *Mccp* vaccine induced seropositivity in majority of the inoculated goats. The finding in the present study was in agreement with that of Tarekegn et al. (2012) who reported seroconversion in 68.4% of vac cinated goats using inactivated CCPP vaccine produced by NVI (Ethiopia). Seroconversion indicates that sufficient *Mccp* antigen and adjuvant were present in the vaccine to induce the proper response. Similar finding was reported by Peyraud et al. (2014) where goats vaccinated at CIRAD with a batch of the reference vaccine displayed rapid marked seroconversion, that was detectable after as little as one week.

While considering only those goats which were negative for *Mccp* specific antibodies in the pre-vaccination sera 48.8% goats seroconverted following vaccination. However, sera samples collected from 51.6% vaccinated goats remained seronegative for *Mccp* specific antibodies 3 weeks post-vaccination. In the present study, blood samples were collected only once after vaccination hence those goats that possibly became seropositive after the third week of vaccination may have been missed from the result. On the other hand, Peyraud et al. (2014) have reported that vaccine quality directly affected the intensity and duration of seroconversion. From their study, lower antigen content and the use of smaller amounts of adjuvant resulted in weaker responses. Thus, quality control is particularly important for vaccines, as the industrial product used is generated on a large scale and may behave differently from the original laboratory product for which efficacy was demonstrated (Rurangirwa et al., 1987a, b).

In addition, the management situation at the time of vaccination (before and after vaccination) is important for a very good immune response (antibody production). Vaccines should be administered at times of low stress and several weeks prior to expected changes in management that may increase stress or exposure to infectious agents. Good nutrition, both in protein and energy as well as trace minerals and vitamins is required for an adequate immune response (Rashid et al., 2009). With the present field trial, study animals were owned and managed by several pastoralists in the area; hence, vaccinated goats were not managed under uniform and controlled field environment. Thus, several factors like poor nutrition, movement over long distances, high environmental temperatures and immunosuppressive concurrent diseases may have interfered with ability of the animal to mount a good immune response following field vaccination.

The differences in post-vaccinal seroconversion among the age categories were statistically significant. Significantly lower number of positive reactors was recorded among old age goats when compared with young and adult age goats. This observed variation in vaccine responses of the old aged goats might be due to individual genetic variations, health status and other external factors. In addition, several studies have indicated that age-related changes in the immune system may hamper successful vaccination and currently development of vaccination strategies that are effective in all age groups is an important area of research (Weinberger et al., 2008).

Vaccination programs are designed to protect an animal

from infection; however, depending upon the age and health of the animal, vaccination may not stimulate a protective humoral response. It is possible that, as in the human being and other animals, old aged goats may be less responsive than their younger counterparts to current vaccination protocols. In human being, several studies have reported that ageing of the immune system (immunosenescence) contributes to the increased susceptibility of the elderly to infectious disease and to the poor outcome of vaccination (Pawelec, 2007). Vaccination could protect them against several infectious diseases, but it can be effective only if cells that are capable of responding are still present in the repertoire. Recent vaccination strategies in the elderly might achieve low effectiveness due to age-related immune impairment. Immunosenescence affects both the innate and adaptive immunity (Ongradi and Kovesdi, 2010).

It is now becoming apparent that the immune system undergoes age-associated alterations, which accumulate to produce a progressive deterioration in the ability to respond to infections and to develop immunity after vaccination (Weinberger et al., 2008). While these changes have been extensively documented in humans and mouse models, little is known regarding the effect of ageing on the immune response to vaccination in the goat population. Vaccination remains to be the most cost-effective measure for preventing and reducing the severity of infectious diseases like CCPP in the goat population. Past trials have demonstrated that immunization of goats with inactivated CCPP vaccine confers protective immunity; however, all of those trials used approximately equal aged goats for their studies (Rurangirwa et al., 1991; Ayelet et al., 2007; Tarekegn et al., 2012). Hence, further investigation is needed regarding the effectiveness of currently used CCPP vaccine in all (various) age groups of goat population.

In conclusion, serological findings indicated that CCPP is one of the major goat health problems in Borana pastoral area. Therefore, appropriate disease prevention and control strategies should be designed and implemented to mitigate the disease impact.

Field vaccination of goats by inactivated *Mccp* vaccine induced seropositivity (seroconversion) in majority of the inoculated goats. However, it is difficult to establish a correlation between this level and protection. Hence, it would be valuable to conduct CCPP challenge experiment, first under controlled laboratory condition, and then under field level.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Epidemiology and public health significance of bovine tuberculosis in and around Sululta District, Central Ethiopia

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A cross-sectional study was conducted on 858 dairy cows, 1107 slaughter animals and 58 dairy workers in and around Sululta District to investigate the epidemiology and public health importance of bovine tuberculosis (BTB). To that end, comparative intradermal tuberculin test (CIDT), post-mortem examinations, bacteriological analysis, molecular typing and questionnaire survey were employed. The herd and individual animal level prevalence were 11.4% (98/858) and 20% (9/45), respectively. The individual animal prevalence was affected by farming system, herd size, management system, sex, age, breed and body condition (P<0.05). Abattoir survey showed a prevalence of BTB to be 3.5% (39/1107) based on suspicion of tuberculous lesion. Culture positivity in primary culture media was confirmed in 7.7% (3/39) of tissue samples, 11.1% (5/55) of milk samples and 2.5% (1/40) of nasal swab samples. Genus typing of the nine positive isolates indicated that only 11.1% (1/9) one isolate was positive for the genus Mycobacterium. Among the farm attendants, only 6.9% (4/58) of the farm attendants had awareness on the existence of BTB, 10.3% (6/58) had awareness that milk and meat could be a source of BTB and 79.3% (46/58) had habit of raw milk and raw meat consumption. The study reveals the importance of BTB and poor awareness on the existence, source and transmission of the diseases in the study area call for urgent intervention. Conventional preventive measures and large scale collaborative action to design cost effective preventive and control measures at national level is recommended.

Key words: Bovine tuberculosis, cattle, comparative intradermal tuberculin test (CIDT), epidemiology, public health, Sululta, Ethiopia.

INTRODUCTION

Bovine tuberculosis (BTB) is chronic infectious disease of cattle caused by intracellular bacterium, *Mycobacterium bovis* characterized by progressive granulomatous lesions or tubercle in the lung tissues, lymph nodes

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and other organs. *M. bovis* is a member of the *Mycobacterium tuberculosis complex* (MTC). The complex encompasses *M. tuberculosis, M. bovis, Mycobacterium microti, Mycobacterium canetti, and Mycobacterium africanum* (Smith et al., 2006). Bacteria of the *M. tuberculosis* complex are aerobic, non-motile, non-spore-forming, slow-growing and acid-fast bacilli (Thoen et al., 2004).

M. bovis is the most ubiquitous pathogen among mycobacterium species. It has been known to infect many vertebrate animals of all age groups and humans although cattle, goats and pigs are found to be most susceptible (Radostits et al., 2000; Thoen et al., 2006). Among MTC, *M. bovis* has a wide range of target organs (lungs, gastrointestinal tract, mammary gland, kidney and reproductive organs) as well as mammalian hosts (wild animals, domestic animals and humans) (Phillips et al., 2002).

Infection of cattle with *M. bovis* constitutes a human health hazard and economic implications in terms of trade restrictions and productivity losses (Phillips et al., 2002; Villarreal-Ramos et al., 2003). Infected animal loses 10 to 25% of their productive efficiency (Radostits et al., 2000). Direct losses due to the infection become evident by decrease in 10 to 18% milk and 15% reduction in meat production (Radostits et al., 2000).

Human tuberculosis caused by *M. bovis* is becoming increasingly important in developing countries where humans and animals share the same microenvironment and waterholes (Cosivi et al., 1998). It is estimated that in countries where pasteurization of milk is rare and bovine tuberculosis is common, 10 to 15% human cases of tuberculosis are caused by *M. bovis* (Cosivi et al., 1998; Ameni et al., 2007).

The existence of BTB in Ethiopian cattle has long been documented in different parts of the Ethiopia and it is one of the major constraints to the country's socio economic development (Ayele et al., 2004). Various previous fragmented studies have shown that its prevalence range from 0.02-7.96% by abattoir survey while it varies from 4.7-68.6% by tuberculin test in cattle from different parts of Ethiopia (Shitaye et al., 2006).

Sululta is one of the towns located in North Shewa zone of Oromia Regional State having large number of dairy sector that supply milk to a large communities in the Addis Ababa and its suburb. However, there is no information on the epidemiology and public health significance of BTB in the district. Therefore, this study was aimed to investigate the epidemiology of BTB and its public health significance in Sululta District.

MATERIALS AND METHODS

Study site

The study was conducted in and around Sululta District, part of the Oromia National Regional State, Central Ethiopia. Sululta District, representing a highland agro-ecology, is located about 25 km North west of Addis Ababa, the capital of Ethiopia. It is located at 9°3' to 9°31'N latitude and 38°29' to 38°58'E longitude. It has a total area of 1587 km^2. The District is inhabited by 129,000 people of which 64,516 are men and 64,484 are women; 15,145 or 11.74% of its population are urban dwellers. The district is one of the main dairy sheds of Addis Ababa. The district livestock population is estimated at 210,210 cattle, 80,900 sheep, 16,491 goats, 32,862 equines and 75.936 poultry (CSA, 2007).

Study population

The study populations were dairy cows, slaughtered animals and farm attendants in the study area.

Study design and sampling

A cross sectional study design with census (from all volunteer farm owners) sampling technique was employed to sample 858 dairy cows for CIDT from 45 dairy farms, 1107 slaughter animals for abattoir survey and 58 farm attendants for questionnaire survey. Sample size for dairy cows was determined based on the expected prevalence of 13.5% obtained by Ameni et al. (2007) in central Ethiopia according to Thrusfield (2007) by increasing the size fourfold for better precision. Purposive sampling was used for the abattoir survey based on the accessibility and availability of logistics as many animals as possible during the study period. The sample size of farm attendants was determined on the basis of the number of farm attendants and farm size included in the study. Furthermore, 55 milk samples from tuberculin reactor milking cows, 40 nasal swabs from dry cows, heifers and calves and 39 tissue samples from lymph nodes, liver, kidneys and lungs containing suspected TB lesion were collected.

Comparative intradermal tuberculin test

Purified protein derivatives (PPDs), which are crude proteins extracted from both bovine and avian mycobacteria were used. For the CIDT test, two sites on the right side of the mid-neck, 12 cm apart in a horizontal line were shaved, cleansed with a swab immersed in 70% alcohol and dried and the initial skin thicknesses were measured with calipers. One site was injected into the upper site with an aliquot of 0.1 ml containing 2,500 IU/ml PPD-A (avian tuberculin, Lelystand, Biologicals BV, Lelystand, the Netherlands). Similarly, 0.1 ml of 2,500 IU/ml PPD-B (bovine tuberculin, Lelystand, Biologicals B, Lelystand, Netherlands) was injected into the lower site. After 72 h, the skin thicknesses at the injection sites were measured and registered. Test results were interpreted according to the recommendations of the Office International des Epizootics (OIE, 2009).

Post-mortem examination

Detailed post-mortem examination was performed on lymph nodes, liver, kidneys and lungs for the investigation of tubercle lesion following the procedure described by Corner (1994). Incision was made into the parenchyma of the lung and other organs that was suspected to contain tuberculous lesions. The tuberculous lesions were cut into slices of about 2 cm using separate surgical blades. The slices were then examined for the presence of suspected tuberculous lesions and samples collected for mycobacteriological culture.

Isolation of Mycobacteria

Milk and nasal swabs from tuberculin positive and tissue samples from suspected tuberculous organs were collected aseptically and processed for isolation of mycobacteria according to the standard methods described by Roberts et al. (1994). The nasal swab samples were first stirred well to release the samples from the swab and centrifuged at 3,000 rpm for 15 min and mixed with an equal volume of 4% NaOH, concentrated by centrifugation and the sediments neutralized with 1% (0.1 N) HCl as described by WHO (2002). Neutralization was checked by using phenol red as indicator and the solution was said to be neutralized when the color changed from purple to yellow. Thereafter, the sediment was cultured using conventional Löwenstein-Jensen (LJ) egg slant medium containing 0.6% sodium pyruvate and glycerol. Then, the culture was incubated at 37°C for 12 weeks and examined on weekly basis for the presence of mycobacterial colonies. Similar procedure was used for culturing of milk samples and tissue samples being tissue samples were homogenized prior to culturing. All the laboratory activities were carried out under Safety Cabinet world class II. Cultures were considered negative if no visible growth was detected after 12 weeks of incubation. In the presence of visible growth of colonies, microscopic examination of cultures using the Ziehl-Neelsen (ZN) staining method was performed to select acid-fast bacilli (AFB) positive isolates. AFB confirmed positive cultures were sub cultured on other LJ media and the isolates killed in water bath at 85°C for 45 min. Then, the heat killed isolates were kept at -20°C for molecular analysis.

Molecular typing

Multiplex polymerase chain reaction (m-PCR) was used to confirm the presence of genus Mycobacterium in the isolate and to differentiate *M. tuberculosis* complex from *M. avium* complex, and other mycobacterial species following the procedure developed by Wilton and Cousins (1992). Heat killed AFB positive samples were used as source of DNA template. DNA amplification was done in a thermocycler with 20 µl reaction volumes consisting of 5 µl of genomic DNA as a template, 8 µl HotstartTaqMaster and ($MgCl_2$, dNTP, Taq polymerase), 0.3 µl of forward and reverse primers for each sample. The primers used were MYCGEN-F, 5' AGA GTT TGA TCC TGG CTG AG 3' (35 ng/µl); MYCGEN-R, 5' TGC ACA CAG GCC ACA AGG GA 3', (35 ng/µl); MYCAV-R, 5' ACC AGA CAT GCG TCT TG 3', (35 ng/µl); MYCINT-F, 5'-CCT TTA GGC GCA TGT CTT TA 3', (75 ng/µl); TB1-F, 5'-GAA CAA TCC GGA GTT GAC AA 3', (20 ng/µl); TB1-R, 5'-AGC ACG CTG TCA ATC ATG TA 3; (20 ng/µl) and 5.2 µl per sample of Qiagen water. The PCR reaction was carried out using Thermal Cycler (VMR Thermcycler 732-1200 Leicestershire, UK) based on the following amplification programme: 95°C for 10 min for enzyme actvation; 95°C for 1 min for denaturation; 61°C for 0.5 min for annealing; 72°C for 2 min for extension, involving 35 cycles all in all; and final extension at 72°C for 10 min. *M. avium*, *M. tuberculosis* and *M. bovis* strain NCTC 8559, H37 Rv and 2122/97 were used respectively as positive controls while H_2O (Qiagen, USA) was used as a negative control. The PCR product was then electrophoresed in 1.5% agarose gel in TAE running buffer 10X containing ethidium bromide at concentration of 0.5 µg/ml in 1.5% agarose gel, 100 bp DNA ladder (USA), and orange 6x loading dye (USA) were used in gel electrophoresis. All members of the Genus Mycobacterium produced a band of 1030 bp where as *M. avium* or subspecies such as *M. avium* sbsp. paratuberculosis, *M. intracellularae* and members of *M. tuberculosis* complex produce a band, 180, 850and 372 bp, respectively.

Questionnaire survey

Farm attendants were interviewed using a pre-structured questionnaire to assess possible associated risk factors for the occurrence and spread of BTB between cattle and people.

Data management and analysis

The generated data were recorded and coded using Microsoft Excel spread sheet (Microsoft Corporation) and analyzed using STATA version 11.0. Multivariate logistic regression was used to analyze the data and to identify the risk factors. The odds ratio (OR) was calculated to assess the strength of association of different risk factors with the prevalence of BTB. The prevalence was calculated by dividing the number of positive reactor or harboring suspected tuberculous lesions cattle by the total number of cattle. P-value less than 0.05 and 95% confidence interval (CI) for odds ratio not including 1 were taken statistically as significant association.

RESULTS

Out of the 45 dairy farms tested by CIDT, 9 (20%) (95% CI: 19.74-20.26%) of them were positive. Among 858 dairy cows tested by CIDT, 98 (11.4%) (95% CI: 9.24-13.56) were positive. Table 1 shows various risk factors affecting the prevalence BTB and the degree of association of each factor with the disease.

Abattoir survey

Out of 1107 cattle slaughtered at Sululta Co-operative Abattoir Enterprise, 3.5% (95% CI: 2.422-4.578) were suspected to have tuberculous lesion. Table 2 shows the distribution and frequency of lesion detection from different lymph nodes.

Isolation of Mycobacteria

Out of 39 tissue samples suspected of having tuberculous lesion, only 7.7% (3/39) were positive for the growth of mycobacteria. Among 55 milk samples cultured from tuberculin-positive lactating cows, only 9% (5/55) were positive for mycobacterial growth. Similarly, out of 40 nasal swab samples collected from tuberculin-positive non-lactating dairy cattle, only 2.5% (1/40) were positive for mycobacterial growth.

Molecular typing

Mycobactreium genus typing was conducted on the 9 culture positive isolates (3 from tissue, 5 from milk and 1 from nasal swab isolates). Multiplex PCR result showed that out of the nine culture positive isolates, only 11% (n=1) isolate were positive for the genus Mycobacterium.

Table 1. Multivariate analysis showing risk factors for the occurrence of BTB at animal level in Sululta District.

Risk factor	No. (%) of animals		OR (95%CI)
	Examined	Positive	
Sex			
Female	649	94(14.5%)	1
Male	209	4(1.9%)	0.11(0.10-0.12)
Age (years)			
<3	302	6%	1
3-5	295	14.2%	0.38(0.36-0.39)
6-8	178	17.98	0.29(0.28-0.30)
>8	61	4.9%	1.23(1.23-1.23)
Herd			
≤15	249	1.2%	1
16-35	359	1.4%	0.19(0.18-0.19)
>35	227	38.5%	1.08 (1.06-1.09)
Breed			
Zebu	111	1.8%	0.12(0.12-0.13)
Cross	724	12.8%	1
Body condition			
Good	622	4%	0.17(0.15-0.18)
Medium	134	19.4%	0.18(0.17-0.19)
Poor	79	55%	1
Animal origin			
Out	140	7.9%	1
Farm	695	12%	0.62 (0.60-0.63)
Production system			
Extensive	486	2.1%	1
Semi-intensive	206	4.9%	0.02(0.01'-0.03)
Intensive	143	52.44%	0.45(0.42-0. 49)
Management system			
Good	199	0.5%	1
Medium	493	3.9%	0.04(0.04-0.05)
Poor	143	52%	0.13(0.12-0 .13)

Questionnaire survey

From fifty-eight farm attendants interviewed, only 6.9% (4/58) had awareness on BTB and knew the transmission of TB from cattle to humans and vice versa and 10.3% (6/58) knew that milk and meat are sources of BTB) (Table 3). Among the farm attendants, 79.3%(46/58) had habit of raw milk and raw meat consumption (Table 4). Only 10.3% (6/58) of the respondents consume and feed their families and babies on boiled milk. Large number, 72.4% (42/58) of the respondents shared the same shelter with farm cattle.

DISCUSSION

In the present study, animal and herd level prevalence of 11.4 and 20% were recorded in dairy herds using CIDT-test. These findings are in agreement with the study conducted in other parts of the country (Fikre, 2011). Although, the finding is contrary to the study conducted by other researchers (Elias et al., 2008, Tsegaye et al., 2010). In addition, the animal prevalence reported in the present study is much lower than those reported

Table 2. Distribution and frequency of suspected tuberculous lesion in cattle slaughtered at Suluta.

Anatomic site	Frequency	Percent (%)
Retropharyngeal LN	5	12.82
Apical LN	2	5.13
Bronchial LN	2	5.13
MandibularLN	2	5.13
Mediastenal LN	11	28.21
Mesentric LN	7	17.95
Intestinal LN	10	25.64
Total	39	100

LN = Lymph nodes.

Table 3. Farm attendants' awareness of bovine tuberculosis and its mode of transmission.

Farm attendants' knowledge	Number of respondents
Awareness of BTB	4 (6.9%)
Know that cattle transmit BTB to humans	4 (6.9%)
Know that humans transmit TB to cattle	4 (6.9%)
Know that milk and meat are source of infection	6 (10.3%)

Table 4. Milk and meat consumption habit of farm attendants

Consumption habit	Number	Percent
Milk drinking		
Raw milk	46	79.3%
Only boiled milk	6	10.3%
Not drinking	6	10.3%
Meat eating		
Only cooked meat	4	6.9%
Raw meat	46	79.3%
Not eating	8	13.8%

earlier by other workers (Ameni et al., 2003b; Shitaye et al., 2006). On the other hand, the animal prevalence recorded in the present study is much higher than the animal prevalence reported (Tschopp et al., 2009, 2010) in various parts of Ethiopia.

The differences among various studies might be attributed to the differences in herd sizes, age, body condition, sex, production system, breed of animals and the sample size considered. In the present study, majorityof the farms included have smaller herd sizes while majority of the previous studies were conducted on farms with relatively larger herd sizes. O'Reilly and Daborn (1995) have shown that the transmission of BTB from cattle to cattle is largely influenced by herd size, that is, the larger the herd size, the greater the chance of transmission. In Tschopp et al. (2009, 2010) studies, only Zebu breed that managed under extensive traditional cattle management system were included in the study where as in the present study, both cross and zebu breeds cattle managed under semi-intensive and intensive cattle production systems were included in the study. Substantiating the observations of the present study, it was previously documented that the prevalence of BTB is affected by cattle breed and husbandry (Radostitis et al., 2000).

Cattle groups with age greater than eight years were exposed 1.23 times than those with age of three years, to BTB. As reported by Radostits et al. (2000), as age increases, the probability of acquiring TB infection increases. The higher prevalence of BTB observed in female cattle than in male cattle could be due to the small number of male cattle in dairy farms. It may also be due

to the greater productivity stress and longer life span among female animals. In agreement with reports of Elias et al. (2008), body condition was associated with tuberculin reactivity. Similarly, animals kept under poor management system were two times more likely to be tuberculin test positive than those managed under good management system. Similar to the observation of the present study, previous studies also reported higher prevalence in animals with poor body condition as compared to those with good body condition scores (Cook et al., 1996; Kazwala et al., 2001; Asseged et al., 2004; Fikre, 2011). However, it is difficult to decide whether BTB has caused poor body condition or animals in poor conditions were susceptible than those in good body condition.

Abattoir inspection of carcasses revealed a prevalence of 3.52%. This is consistent with the report of Jemale (2005) and Ameni and Wudie (2003). In this study, the lesions were predominantly found in mediastenal lymph node which is consistent with the previous findings of Ameni and Wudie (2003), Asseged et al. (2004), Teklu et al. (2004) and Fikre (2011). A number of studies revealed that the majority of TB lesions are located in the thoracic cavity suggesting the inhalation route being the principal route of BTB transmission (Corner, 1994; Ameni and Wudie, 2003; Phillips et al., 2003; Teklu et al., 2004). In contrast, Cleveland et al. (2007) reported that 61.3% of carcasses in Tanzania had lesions in the gastrointestinal tract.

Out of 39 tuberculous lesions cultured, culture positivity in primary culture media was confirmed only in 5.12% (2/39), which was inconsistent with a report by Fikre (2011), who reported 44.2% culture positivity. Of these tissue samples, only 2.6% (1/39) were positive for genus Mycobacterium by multiplex PCR. This lower isolation rate of mycobacteria may have resulted from reduced sensitivity of culture arising from prolonged storage at field sites, and freeze-thaw cycles that occurred during transportation and contamination of tissue samples and overgrowth of *M. bovis* with environmental mycobacteria (Cleveland et al., 2007). On the other hand, out of 55 milk samples cultured, 9% (5/55) growth was observed. This was different from the findings (Yalelet, 2010; Hussein, 2009) in which no growth of *M. bovis* were observed in milk of tuberculin skin test positive cows. However, of the 55 milk and 40 nasal swab samples cultured, no isolate was confirmed to be a member of the genus Mycobacterium by multiplex PCR. This result is different from those reported by Fikre (2011) and Marlia et al. (2013) who reported 25 and 8% of the isolates were members of the genus Mycobacterium, respectively.

Among farm attendants interviewed, only 6.9% knew about BTB and its transmission from cattle to humans and vice versa. Close physical contact between the owner and cattle and the consumption of raw milk or milk products facilitate the transmission of BTB (Cosivi et al., 1998; WHO, 1993). Among the farm attendants, 79.3% (46/58) had habit of raw milk and raw meat consumption and only 10.3% (6/58) of the respondents consume and feed their families and babies on boiled milk. This finding was relatively consistent with the findings of Fikre (2011) who reported 94.2% of the interviewed households had habit of raw milk and milk products (yoghurt) consumption. This consumption habit might expose the public to risk of acquiring tubercle bacilli from animal (Cosivi et al., 1998). In conclusion, this study demonstrated the importance of BTB and various possible risk factors for the disease and its implication for public health in the Sululta District. The majority of the farm attendants in the area was not aware of the existence of BTB, its mode of transmission and had habit of raw milk and raw meat consumption behavior which is a potential risk factor for the transmission of the disease call for urgent need of intervention.

The authors recom-mended application of general conventional preventive measures such as improving farm management system, public awareness about the disease, its mode of trans-mission, importance of boiling or pasteurization of milk, adequate cooking of meat and strict meat inspection. Furthermore, on large scale, collaborative work is needed to establish clearly the impact of the disease on production and its zoonotic importance so as to design cost effective preventive and control measures at national level.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Antimicrobial activity of *Rosa damascena* petals extracts and chemical composition by gas chromatography-mass spectrometry (GC/MS) analysis

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Antimicrobial activity of alcoholic and aqueous extracts from *Rosa damascena* was evaluated against 10 pathogenic microorganisms. Minimum inhibition concentration (MIC), Minimum bactericidal concentration (MBC) and the diameter of inhibition zone (DIZ) were determined by *in vitro* bioassays using hole-plate diffusion method and broth micro-dilution method (BMD) against *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus, Pseudomonas aeruginosa* ATCC 27853, *Psuedomonas aeruginosa*, *Escherichia coli* ATCC 25922, *E. coli*, *Streptococcus pneumoniae* ATCC 55143, *Acinetobacter calcaoceuticus, Salmonella enteritidis* and *Aspergillus niger* ATCC 16404. While hexane extracts showed very low activity against the test microorganisms, ethanol, methanol and water extracts significantly exhibited antimicrobial activity and inhibited the growth of Gram-positive and Gram-negative bacteria as well as *A. niger* at all tested concentrations. The most active antimicrobial effect was recorded for ethanol extract of *R. damascena* against *P. aeruginosa* ATCC 27853 at MIC and MBC of 62.5 µg/ml (DIZ = 34 mm), *E. coli* ATCC25922 at MIC and MBC of 62.5 µg/ml (DIZ = 30 mm). MIC and MBC data obtained from the antimicrobial studies were analyzed for significant difference at $p<0.05$ using one way analysis of variance (ANOVA). The extracted oil of Damascus rose petals were characterized by GC/MS; analysis reported that 30 compounds were present. The predominant components were citronellol (14.8- 29.0%), geraniol (11.3-16.2%) and nerol (11.6%) while the phenyl ethyl alcohol was 1.2%. This study sheds the light on the efficacy of plant extracts to combat pathogens which will help as natural antimicrobial agents.

Key words: *Rosa damascena*, antimacrobial activity, gas chromatography-mass spectrometry (GC/MS), Rose oil extracts.

INTRODUCTION

Infectious disease account for approximately one half of all deaths in tropical countries (Iwu et al., 1999) and they are considered a major threat to human health because of the unavailability of vaccines or limited chemotherapy. Ranked 5th in 1981, infectious diseases continue to be a growing public health concern (Kone et al., 2006) and

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they became the 3rd leading cause of death in 1992, with an increase of 58% (Pinner et al., 1996). Most of the current antibiotics have considerable limitations in terms of antimicrobial spectrum, side effects and their widespread overuse has led to increasing clinical resistance of previously sensitive microorganisms and to the occurrence of uncommon infections (Cos et al., 2006). Plants used for traditional medicines contain a wide range of substances that can be used to treat chronic as well as acute infectious diseases (Diallo et al., 1999; Ofokansi et al., 2011), and have therefore become sources of important drugs and the pharmaceutical industries have come to consider traditional medicine as a source of bioactive agents that can be used in the preparation of synthetic medicines.

Rosa damascena mill L., commonly known as Damask rose (Kaul et al., 2000), is one of the most important species of Rosaceae family. Rosaceae are well known ornamental plants and have been referred to as the king of flowers (Cai et al., 2005; Nikbakht et al., 2005). At present time, over 200 rose species and more than 18000 cultivars form of the plant has been identified (Gudin, 2000). There are evidences that Rosaceae family is an ancient plant (Nikbakht and Kafi, 2008; Yassa et al., 2009). Some fossils of rose are found in America that is 30 million years old (Yassa et al., 2009). The origin of Damask rose is the Middle East, but the origin of its fragrant oil and extracts is Greece (Arezoomandan et al., 2011). The highest quality rose water is produced in Taif Governorate, KSA. Kaaba (God House) in Mecca, Saudi Arabia, is washed yearly by unique and special rose water of Taif. Rose water is also of high value in the food industry and some special foods are prepared using this product (Nikbakht and Kafi, 2008). The *R. damascena* has also been used for medicinal purposes (Hongratanaworakit, 2009). Various products and isolated constituents from flowers, petals and hips (seed-pot) have been studied in a variety of *in vivo* and *in vitro* studies. It has been shown that *R. damascena* has wide spectrum antimicrobial activities. Essential oil, absolute and hydrosol are important products that showed these effects. Ulusoy et al. (2009) and Boskabady et al. (2011) showed that essential oil and absolute have strong antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *B. subtilis*, *Staphylococcus aureus*, *Chromobacterium violaceum* and *Erwinia carotovora* strains.

However, there is no detailed study concerning the antimicrobial activity of *R. damascena* performed so far. Most of the studies related to the damask rose have focused on the growing techniques, harvest time, and physical and chemical properties of oil. Therefore, the purpose of this study was to determine the *in vitro* antimicrobial activity against gram negative and gram positive bacteria as well as the chemical compositions of *R. damascena* volatile oil by using GC/MS to determine the major constituents of high quality rose oil.

MATERIALS AND METHODS

Preparation of extracts

The present study was conducted to determine the chemical composition (constituents) and antimicrobial activities of *R. Damascena* flower extracts. The flowers were collected in April, 2013 during the rose harvest season, from different local farms (AL-Hada and Al-Shefa), Taif governorate- Saudi Arabia. The green parts of roses were removed and the remains were cut into small pieces. Following the collection, part of the flowers was immediately frozen in closed containers at -80°C for further analysis. The rest of the flowers, 0.5 kg of fresh flowers from each accession were used immediately for alcoholic and water extraction. Fresh and dried parts of *R. damascena* (petals, flowers and seed pods) were either powdered or soaked freshly in five volumes of 50% ethanol, 80% methanol, and 100% hexane by stirring overnight, filtered through Whatman No. 1 filter paper after 72 hand centrifuged at room temperature at 5000 x g. The supernatants were evaporated to dryness at 45ºC under reduced pressure. Powdered *R. damascena* (500 g each) were extracted twice overnight with 2500 ml of distilled water at room temperature. The supernatant were collected and evaporated to dryness at 45ºC under reduced pressure. The yield of each extract was adjusted to be less than 10% (20 g of each alcoholic extract was dissolved in 100 ml distilled water containing 30% DMSO to get concentration 500 µg/ml) and stored in dark sterile bottles and became ready until use. The reconstituted alcoholic extracts were sterilized by filtering through 0.45 µm membrane filter and tested for sterility after membrane filtration by introducing 2 ml of the extract into 10 ml of sterile nutrient broth and incubated at 37°C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period (Ronald, 1995).

Test microorganisms

A total of 10 microbial cultures belonging to nine bacteria and one fungus species, were used in this study. These microorganisms included *E. coli* (clinical isolet ,ur), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *P. aeruginosa* (clinical isolet, ETT), *S. aureus* (ATCC 25923), *S. aureus* (clinical isolet, Nasal), *S. pneumoniae* (ATCC55143), *S. enteritidis* (ATCC 13076), *A. calcaoceuticus* (clinical isolet, ur) and *A. niger* (ATCC 16404). These microorganisms were obtained from the Microbiology Unit, Al-Edwani Hospital, Taif Governorate, KSA. The organisms were maintained on nutrient broth, nutrient agar (Biolab, Hungary), potato dextrose agar and potato dextrose broth. The antibacterial assays were carried out using Mueller Hinton II Agar (Oxoid, England) and broth. The antifungal assays were carried out using, potato dextrose broth and potato dextrose agar.

Antimicrobial activity

All plant extracts were dissolved in sterile distilled water containing 30% DMSO to a final concentration of 500 µg/ml and sterilized by filtration by 0.45 µm Millipore filters. Antimicrobial tests were carried out in this report by agar well diffusion assays according to the NCCLS citeria. The antibacterial activity was carried out by employing 24 h cultures of *E. coli* (ur), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *P. aeruginosa* (ETT), *S. aureus* (ATCC 25923), *S. aureus* (Nasal), *S. pneumoniae* (ATCC55143), *S. enteritidis* (ATCC 13076), *A. calcaoceuticus* (ur) and *A. niger* (ATCC 16404). Activity of aqueous and alcoholic extracts of *R. damascena* (RD) was tested separately using Agar well diffusion method (Perez et al., 1990; Murray et al., 1995; Olurinola, 1996; Srinivasan et al., 2001; Dulger and Gonuz, 2004). The medium

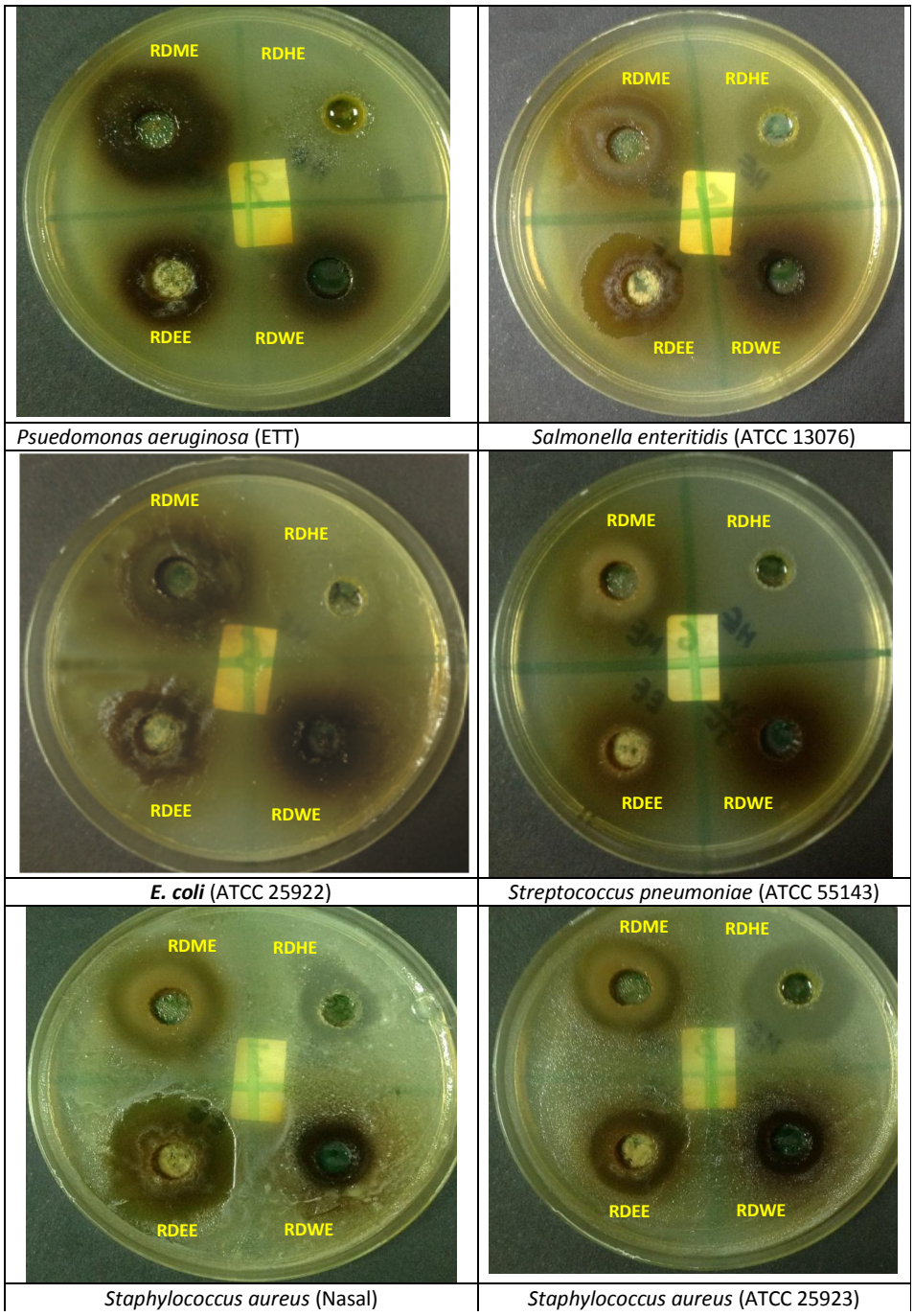

Figure 1. Agar well diffusion assay showing the antibacterial activity of different extracts of RD. Each well containing 100 µg of the each extract.

was sterilized by autoclaving at 120°C (15 lb/in^2). About 30 ml of the agar medium with the respective strains of bacteria was transferred aseptically into each sterilized Petri plate. The plates were left at room temperature for solidification. A well of 6 mm diameter was made using a sterile cork borer. Antibacterial assay plates were incubated at 37±2°C for 24 h. The diameter of the zone of inhibition was measured. Sterile ultrapure water and DMSO were used as negative control. Samples were tested in triplicate and results are expressed as mean ± standard deviation.

Determination of antifungal activity

The agar well diffusion method (Anesini and Perez, 1993) was modified. Potato dextrose agar (PDA) was used for fungal cultures (Figure 1). The culture medium was inoculated with the fungal strain *A. niger* ATCC 16404 separately suspended in potato dextrose broth. A total of 6 mm diameter wells were punched into the agar and filled with plant extracts and solvent blanks (ethanol, methanol and hexane). Standard antibiotic (Nystatin, concentration 1 mg/ml)

was used as positive control and fungal plates were incubated at 28°C for 48 h. The diameters of zone of inhibition observed were measured. Antifungal activities were determined by measuring diameter of inhibition zone (DIZ) in mm. Each experiment was repeated thrice and the average values of antimicrobial activity were calculated (Shahidi, 2004).

Broth micro-dilution method (BMD)

MICs and MBCs of *R. damascena* extracts were determined by using BMD method as described by the NCCLS in flat-bottomed 96-well clear plastic tissue-cultured plates (NCCLS, 2003a). The MIC was assayed using two-fold BMD method in MH Broth in 96-well plates. Plates contained two fold dilutions of antimicrobial agents at the concentration ranges: 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/mL. 1% DMSO was used as a solvent control. Plant extracts were diluted to twice the desired initial test concentration (1 mg) with Muller Hinton broth (MHB) (Oxoid, UK). All wells, except the first were filled with MHB (50 µL). Plant extract (100 µL) was added to the first well and serial two-fold dilutions were made down to the desired minimum concentration (32 µg). An over-night culture of bacteria suspended in MHB was adjusted to turbidity equal to 0.5 McFarland standards. The plates were inoculated with bacterial suspension (50 µL/ well) and incubated at 37°C for 24 h. Then the turbidity was observed visually by eyes. MICs and MBCs were determined for ethanol, methanol, hexane and aqueous extracts of *R. damascena*. MIC was determined as the lowest concentration of plant extract that inhibit the growth of each microorganism. MBC was determined as the lowest concentration of plant extract that prevent the growth of bacteria after sub-culturing on MH agar plates. To determine the MBCs, the suspensions (20 µl) were taken from each well without visible growth and inoculated in MH agar for 24 h at 37°C. To determine the Minimal Fungicidal Concentration (MFC), 100 µL of each dilution showing no growth was spread on PDA. The inoculated Petri dishes were incubated at 37°C for 48 h for fungal cultures. Tests were performed in triplicate for each test concentration.

Isolation of volatile oil

One hundred grams of dried flowers of each plant were ground in a blender and then subjected to hydrodistillation for 3 h according to the standard procedure described in the European Pharmacopoeia (2004). The oil was solubilized in *n*-hexane, dried over anhydrous sodium sulphate and stored at +4°C in the dark until tested and analyzed.

Gas chromatography-mass spectrometry (GC/MS) analyses

The essential oils were analyzed on a gas chromatograph (GC)-mass spectrometer (MS); Clarus 500 GC/MS (PerkinElmer, Shelton, CT) was used. The software controller/integrator was TurboMass, version 4.5.0.007 (PerkinElmer). An Elite-1 GC capillary column (30 m, 0.25-mmID, 0.25 DF, PerkinElmer) was used. The carrier gas was helium (purity 99.9999%) at a flow rate of 0.9 mL/min (initial 7.6 p.s.i. flow initial 36.2 cm/s, split; 50:1). Temperature conditions were: inlet line temperature, 270°C; source temperature, 210°C; trap emission, 100°C; and electron energy, 70 eV. The column temperature program was: 80°C hold for 5 min, increased to 150°C (rate, 5°C/min), and held for 5 min, increased to 270°C (rate, 20°C/min) and hold for 5 min. The injector temperature was 220°C. MS scan was from 45 to 350 *m/z*.

A sample volume of 50 µL was diluted with 500 µL of chloroform, vortexed for 2 s, and a volume of 1 µL was injected for GC-MS analysis. A volume of 50 µL from this diluted sample was transferred to total recovery vial 1-mL, mixed with 50 µL of MSTFA, capped, vortexed for 2 s, heated at 80°C for 30 min using heating block for half insertion of vial, cooled, and a volume of 1 µL was injected for GC-MS analysis.

The total ion chromatogram (TIC) was recorded from 45 - 350 *m/z*. The targeted peaks were extracted by the knowledge of major *m/z* fragments, averaged masses at peak top, and searched for matched compounds using mass spectrometry data bank NIST2008 database. The percentage composition of the essential oil was computed by the normalization method from the GC peak areas measurements.

Statistical analysis

Comparison of data was performed using the one way ANOVA and is presented as mean ± standard deviation. Comparison of MIC and MBC values, tests were made in triplicate for quantification. Values of $p<0.05$ were considered significant.

RESULTS

Antimicrobial activities of plant extracts

The results show that *Escherichia coli* ATCC 25922 was sensitive to all antibiotic disks tested but resistant only to AMC, *P. aeruginosa* ATCC 27853 was resistant to all antibiotic tested but sensitive only to CEZ. The rest of tested bacterial isolates were sensitive to all antibiotic disks tested. The antibacterial activity of aqueous and alcoholic extracts of flowers petals of *R. damascena* were observed using agar well diffusion method by measuring the diameter of the growth inhibition zone (Table 1).

The ethanolic extracts of *R. damascena* (RDEE) showed a positive significant antibacterial activity against all tested bacteria; diameter of zone of inhibition ranged between 15 mm for *A. niger* ATCC 16404 to 34 mm for *P. aeruginosa* (ATCC 27853). Methanolic extracts of *R. damascena* (RDME) showed moderate degree of activity against most bacterial organisms tested (diameter of zone of inhibition ranged) between 13 mm for *A. niger* ATCC 16404 to 25 mm for *Psuedomonas aeruginosa* (ATCC 27853) and *Salmonella enteritidis* (ATCC 13076), while the hexane extracts (RDHE) showed very less effect against tested bacteria. The ethanolic extracts of RDEE showed a highly significant antibacterial activity against *P. aeruginosa* (ATCC 27853), and *E. coli* (ATCC 25922).

Hexane extracts of *R. damascena* (RDHE) showed the lowest degree of inhibition against most of the tested bacteria; diameter of zone of inhibition ranged between 9 mm for *A.niger* ATCC 16404 to 13 mm for *Psuedomonas aeruginosa* (ATCC 27853). The antifungal activity was determined by measuring the diameter of zone of inhibition recorded. RDEE, RDWE and RDME were found to have maximum antibacterial and antifungal activity in comparison to hexane extracts. RDEE possessed potent antifungal activity against *A. niger* showing diameter of zone of inhibition ~ 15 mm while methanolic extracts and Water extracts showed diameter

Table 1. MICs, MBCs and MFCs in µg/mL of RD extracts against nine bacteria and one fungal isolate.

Name of the organism	Test sample	DIZ (mm)	MIC	MBC/MFC	±SD
Acinetobacter calcaoceuticus (Ur)	RDEE	27	125.0	250.0	±62.5
	RDME	25	125.0	250.0	
	RDHE	15	250.0	250.0	
	RDWE	24	125.0	125.0	
Escherichia coli (Ur)	RDEE	25	62.50	125.0	± 193.4
	RDME	20	62.50	62.50	
	RDHE	10	500.0	500.0	
	RDWE	25	125.0	250.0	
Psuedomonas aeruginosa (ATCC 27853)	RDEE	**34**	62.50	62.50	±193.4
	RDME	25	125.0	125.0	
	RDHE	13	250.0	500.0	
	RDWE	20	250.0	250.0	
Salmonella enteritidis (ATCC 13076)	RDEE	25	62.50	125.0	±157.2
	RDME	25	250.0	250.0	
	RDHE	10	500.0	500.0	
	RDWE	25	250.0	250.0	
Escherichia coli (ATCC 25922)	RDEE	**30**	62.50	62.50	±235.9
	RDME	20	125.0	125.0	
	RDHE	10	250.0	500.0	
	RDWE	10	500.0	500.0	
Streptococcus pneumonia (ATCC 55143)	RDEE	27	62.50	125.0	± 176.7
	RDME	22	250.0	250.0	
	RDHE	10	250.0	500.0	
	RDWE	25	125.0	125.0	
Staphylococcus aureus (Nasal)	RDEE	**30**	62.50	62.50	±207.2
	RDME	20	125.0	250.0	
	RDHE	11	250.0	500.0	
	RDWE	28	62.50	62.50	
Staphylococcus aureus (ATCC 25923)	RDEE	28	62.50	62.50	±207.2
	RDME	20	62.50	125.0	
	RDHE	11	250.0	500.0	
	RDWE	20	125.0	250.0	
Psuedomonas aeruginosa (ETT)	RDEE	25	125.0	250.0	± 193.4
	RDME	20	125.0	125.0	
	RDHE	10	250.0	500.0	
	RDWE	20	125.0	250.0	
Aspergillus niger (ATCC 16404)	RDEE	15	125.0	250.0	±125.0
	RDME	13	250.0	250.0	
	RDHE	9	250.0	500.0	
	RDWE	13	250.0	250.0	

RDEE, *Rosa damascena* ethanol extract; RDHE, *Rosa damascena* hexane extract; RDME, *Rosa damascena* methanol extract; RDWE, *Rosa damascena* water extract; DIZ, diameter of inhibition zone; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; MBC, minimum bactericidal concentration; ±SD, standard deviation.

of zone of inhibition ~13 mm. Hexane extracts *R*. *damascena* showed very low antifungal activity against *Aspergillus niger* ATTC 16404 (diameter of zone of inhibition of 9 mm).

Water extracts of *R*. *damascena* showed also a highly significant antibacterial activity against tested organisms. The diameters of zone of inhibition ranged from to 13 to 28 mm.

Table 2. Compounds determined in the oil of *Rosa damascena* extracted by hydro-distillation.

Compound identified	Retention Time (min)	Content Average (%)
β-Linalool	5.65	0.012
***Phenyl ethyl Alcohol**	6.82	**1.276**
2.2-Phenylethyl trimethylsilyl ether	8.52	0.275
Benzoic acid trimethsilyl ester	8.87	0.139
***Citronellyl formate**	9.58	**1.421**
2,2,4,7,7-pentamethyl -3,6-Dioxa-2,7-disilaoctane	10.01	0.1943
2,2,11,11- tetramethyl-3,6,10 - Trioxa-2,11-disiladodecane	10.07	0.01
Trimethylsilane (Decyloxy)	10.21	0.335
Bis(trimethylsilyl)-1,2-Butanediol	10.27	0.622
***β-Citronellol**	10.74	**29.013**
***β-Citronellol,trimethylsilyl ether**	10.97	**14.83**
***Nerol, trimethyl ether**	11.4	**11.66**
***Geraniol**	11.49	**11.395**
***Geraniol, trimethyl silyl ether**	12.1	**16.271**
[2-methoxy-4-1 (1-propenyl)phenoxy]trimethyl- Silane	14.33	0.381
Geranyl propionate	17.08	0.24
Eugenol methyl ether	17.33	0.86
Germacrene D	17.46	0.636
Geranyl isobutyrate	18.09	0.054
σCadinene	18.46	0.036
Nerolidol	18.62	0.233
Geranyl isobutyrate	19.14	0.27
Oxalic acid,decyl 2-phenyl ester	19.45	0.497
Elemol	20.14	0.071
trans, trans-Farnesol, trimethylsilyl ether	20.37	0.375
1.γ-Eudesmol	20.7	0.123
4βH,5α-Eremophil-1(10)-ene, 11-(trimethylsiloxy)	25.24	0.023
1.Methyl pimar-7-en-18-oate	29.77	0.642
Methyl abietate	30.04	0.081

*Dominant compounds are indicated in bold

The results of minimum inhibitory concentrations (MICs) revealed that the *R. damascena* alcoholic extracts exhibited the best anti-bacterial activities towards Gram-negative and Gram-positive bacteria with MIC and MBC values between 62.5 to 250 µg/ml (Table 1).

The minimum fungicidal concentration (MFC) of *R. damascena* (RD) extracts in different solvents were evaluated against *A. niger* ATCC 16404. Ethanolic, methanolic and water extracts were found to have maximum antifungal activity in comparison to hexane extracts. Ethanolic extracts of RD (RDEE) showed maximum potency against *Aspergillus niger* ATCC 16404 at highest MFC value of 62.5 µg/ml while the MFC value of ethanolic and methanolic extracts were 125 µg/ml respectively. Thus, the MBC and MFC of *R. damascena* (RD) extracts showed a bactericidal and fungicidal behavior of these extracts. The anti-bacterial activity of *R. damascena* alcoholic and water extracts were higher than that of hexane extract.

In the current study, 0.2 ml of rose oil was obtained from 250 g of dried Taif rose petals processed through steam distillation and the oil obtained was observed to be pale yellow in color, and the yield of essential oil was 0.15% (v/w). GC/MS analysis reported that 30 compounds (Table 2) were present in the Taif rose essential oil of which β-Citronellol (29.013%) was recorded as the major component in rose oil followed by Geraniol, trimethyl silyl ether (16.271), β-Citronellol,trimethylsilyl ether (14.83%), Nerol, trimethyl ether (11.66%), Geraniol (11.395%), Citronellyl formate (1.42%) and Phenyl ethyl Alcohol (1.276%) (Figure 2).

DISCUSSION

Generally, the degree of the plant extract activity is revealed by the size of inhibition zone that is expressed by the diameter of the referred inhibition zone. Due to the

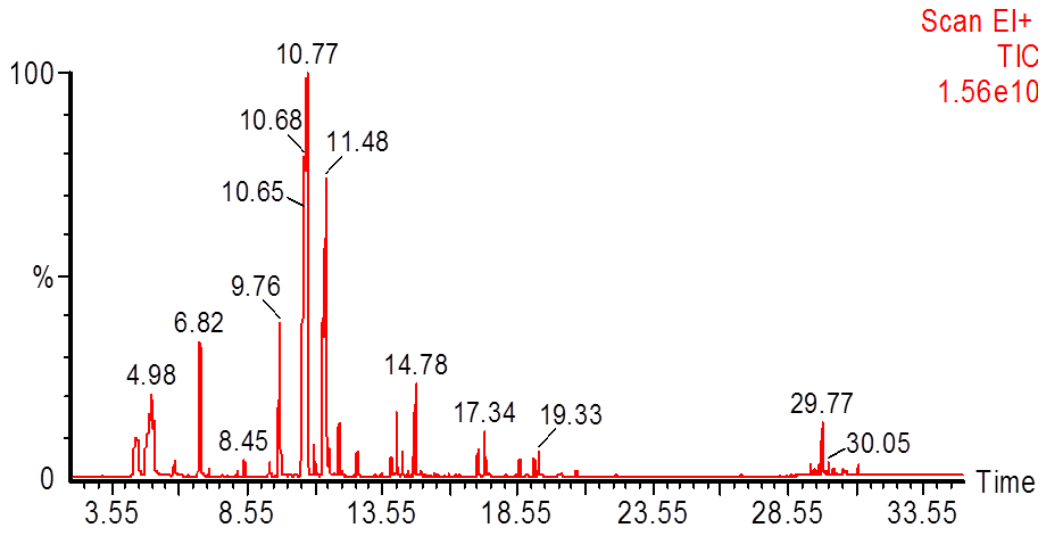

Figure 2. GC/MS chromatogram of *Rosa damascena* essential oil collected from Taif province.

simple nature of this assay, this technique is less suitable for more precise quantification purposes. MIC and MBC values were calculated to determine the susceptibility of a range of microbial species to a particular extract (Faleiro, 2011). The Gram-negative *Pseudomonas aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were the most sensitive strains (MIC of 62.5 µg/ml) for RDEE. Similarly, *Staphylococcus aureus* (ATCC 25923) was the most sensitive strain to RDME (MIC of 62.5 µg/ml). These results agreed with that obtained by Talib and Mahasneh (2010) who reported that butanol extract of *R. damascena* showed the highest activity (100% inhibition) against *S. typhimurium* and *B. cereus* (MIC 0f 62.5 and 250 µg/ml) respectively, while the aqueous extract of *R. damascena* was also active against *C. albicans* (MIC of 125 µg/ml). Also, in a study reported by Ulusoy et al. (2009) in Turkey, it was found that Rose absolute and essential oil had a strong antibacterial activity against *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *B. subtilis* (ATCC 6633), and *S. aureus* (ATCC 6538). Shohayeb et al. (2014) recorded a similar results that the essential oil and different extracts of Taify *R. damascen* possess brood spectram antimicrobial activity against gram positive and gram negative bacteria , acid fast bacteria and fungi with MIC and MBC range from 0.125 to 2 and 0.4 to 0.5 mg/ml, respectively .

The molecular mechanism of action of the *R. damascene* extracts on Gram-negative bacteria is unknown, but the ethanol extracts can probably inhibit the generation of adenosine triphosphate from dextrose and disrupt the cell membrane (Gill and Holley, 2006).

The high amount of hydrocarbons and the concurrent presence of monoterpenes (β-Linalool) could also contribute to inhibit the microbial DNA gyrase as reported by Cushnie and Lamb (2005).

It is well known that *R. damascene* components show also better antimicrobial effectiveness against Gram-positive bacteria. Due to the composition of outer membrane, plant extracts can alter not only such structures but penetrate within the cell, leading to those alterations, such as the denaturation of proteins and enzymes, the “unbalance” of the K+ and H+ ion concentration, until the modification of the entire cell morphology, which can lead to the death of the microorganism (Marrufo et al., 2013).

Phenolic compounds show generally a good antimicrobial effectiveness against Gram-positive bacteria; their effect is dependent on their amount: at low concentrations they are able to interfere with enzymes involved in the production of energy and at higher concentrations, they can induce the denaturation of proteins (Tiwari et al., 2009) until an irreversible modification of the cell and death.

The yield of essential oil was 0.15% (v/w) which agrees with the results of Moeina et al. (2010) who has obtained 0.16% (v/w). Our results of GC/MS analysis reported that 30 compounds were present in the Taif rose essential oil of which β-citronellol (29.013%) was recorded as the major component in rose oil followed by geraniol, trimethyl silyl ether (16.271), β-citronellol, trimethylsilyl ether (14.83%), nerol, trimethyl ether (11.66%), geraniol (11.395%), citronellyl formate (1.42%) and phenyl ethyl alcohol (1.276%).

These results agree with that obtained by Verma et al. (2011) who found that Citronellol (15.9-33.3%), Geraniol (8.3-32.2%), Nerol (4.0-9.6%), nonadecane (5.5-16.0% and heneicosane (2-.6-7.9%) were reported as the major components in rose oil. Also, Ulusoly et al. (2009) reported that Citronellol and Geraniol were the major compounds of Turkish *R. damascena* essential oils. The results of GC/MS of Iranian *R. damascena* reported by Yassa et al. (2009)

showed some similarity to our results in which Linalool (3.68%), Nerol (3.05%), Geraniol (15.5%), nonadecane (18.56%), tricosane (16.68%), and n-hexatriacontane (24.6%) were the major components of *R. damascena.* Babu and Kaul (2005) reported that Bulgarian rose oil composed of Citronellol (30.31%), Geraniol (16.96%), Phenyl ethyl alcohol (12.60%), Nerol (8.46%), hexacosane (3.70%), nonadecane (2.7%), Linalool (2.15%), Ionone (1.00%), ecosane (1.65%), docacosane (1.27%), farnesol (1.36%), neryal acetate (1.41%), citronellyl propionate (1.38%), geraniol (1.35%), pinene (0.60%), myrceen (0.46%), cis rose oxide (0.55%), decanal (0.51%), terpine-4-ol (0.55%), caryophyllene+citronellyl act (0.81%), isoborneol (0.57%), and heptadecane (0.92%).

The comparison of the results with the literature showed significant differences for oils, which can be attributed to ecological factors, genetic differences or the development stages of the plant parts analyzed.

Conclusion

Data obtained in this work could be useful in determining the major constituents of the flower essential oil of *R. damascene* which indicate its high quality. The results of antimicrobial activities of it can contribute to confirm the popular uses of these plants in Saudi Arabia in folk practices and to suggest new practical uses as natural aromatic mouth wash and wound disinfectant.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Yellow fever and dengue fever viruses' serosurvey in non-human primates of the Kedougou forest galleries in Southeastern Senegal

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The potential risk of non-human primates in Senegal to be natural hosts for arboviruses of importance for human has been assessed. A total of 58 wild monkeys, including 14 *Erythrocebus patas* and 44 *Chlorocebus sabaeus,* were trapped at three sites within forest galleries and the nearby village of Ngari, in the Kedougou area, Southeastern Senegal. Blood samples were taken and sera analyzed by enzyme-linked immunosorbent assay (ELISA) for the presence of Yellow Fever (YF) and/or Dengue 2 (DEN-2) reacting antibodies. An overall yellow fever seroprevalence of 22.4% was found, including 5.2% and 17.2% YF IgG positive *E. patas* (3/58) and *C. sabaeus* (10/58) respectively. Three of the positive *C. sabaeus* were trapped near Ngari village, and the others in forest galleries. Also, 12.0% of the primates tested positive including 5.2% of *E. patas* and 6.9% of *C. sabaeus*, all of them were from the forest galleries. Ultimately *Cercopithecidae* act as potential amplificatory reservoir hosts for YF virus and, seroconversion observed within wild *C. sabaeus* and *E. patas* demonstrates also an active DENV-2 virus circulation within non-human primates in Senegal. The present study addresses and discusses new insight of both viruses' natural enzootic cycles.

Key words: Yellow fever, Dengue, monkeys, Senegal.

INTRODUCTION

Yellow fever virus (YFV) and Dengue viruses (DENV) belong to the same *Flavivirus* genus of the *Flaviviridae* family.

There are four DENV serotypes also distinguishable by

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their genome (DENV-1, DENV-2, DENV-3 and DENV-4), all of which can cause dengue fever (DF), and dengue hemorrhagic (DHF) (Gubler, 1997; Bhatt et al., 2013). YFV and DENV belong to the same clade within Flaviviridae. Despite the excellent protection afforded by the worldwide available 17D vaccine, YFV still causes, in unprotected persons, severe and often deadly illness (Nathan et al., 2001). Indeed, outbreaks occur annually in West Africa, and cases are typically underreported. The World Health Organization estimates that 200,000 cases of yellow fever occur worldwide each year, from which there are 30,000 deaths, most of which occurring in West Africa (Mutebi and Barrett, 2002). It still remains an important health risk in sub-Saharan Africa and tropical South America (Vainio and Cutts, 1998; Tomori, 2004). Vaccine coverage is often unreliable, particularly in remote regions, and the risk for outbreaks increases whenever routine vaccination breaks down (Nathan et al., 2001). In Senegal, outbreaks have been recorded and the epidemic risk remains (Thonnon et al., 1998a, b). Dengue fever is now one of the most important arthropod-borne viral diseases in humans, accounting for the largest portion of global mosquito-borne disease morbidity and mortality. There is no licensed vaccine for DENV and control of this disease primarily relies on vector control and community. This disease sickens 50 to 100 million people every year, from which 200,000 to 500,000 cases of potential life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) are reported (Noisakran and Chuen, 2008). Dengue infection can cause a spectrum of illness ranging from mild, undifferentiated fever illness to severe fatal hemorrhagic syndrome. The first phase of the illness can last for up to seven days with high fever, severe headache, retro-orbital pain, arthralgia and rash. In 3 to 5% of DENV infections, severe syndrome occurs, including DHF with hemorrhagic tendencies, thrombocytopenia and plasma leakage, and DSS with all the above criteria plus circulatory failure. DHF and DSS are potentially deadly however, patients with early diagnosis and appropriate therapy can recover without sequelae (Guha-Sapir and Schimmer, 2005). Several investigations have been undertaken in West Africa concerning the natural cycle of DENV- wild mosquitoes - non-human primates but failed to prove a dengue sylvatic cycle. However in South East Asia, limited observations favoring a potential DENV sylvatic cycle have been documented: in the Philippines, Simmons et al. (1931) conducted some experiments in Manila and suspected a dengue sylvatic cycle; in Malaysia, extensive field and laboratory investigations conducted on the ecology of the dengue viruses hypothesized a sylvatic transmission cycle (Rudnick, 1986) and in some other countries of South East Asia, Yuwono et al. (1984) suggested the occurrence of a zoonotic reservoir of infection existing in all the primary tropical forests of Malaysia, Thailand, Vietnam, Cambodia and Indonesia.

In Senegal, serosurveillance programs led within wild monkeys in forested areas of the emergence zone also brought little information about the sylvatic cycle of dengue viruses (Cornet et al., 1984; Saluzzo et al., 1986; Diallo et al., 2003).

From June 2002 to November 2006, we performed a study in order to determine the role of feral monkeys in the sylvatic cycle of DENV. Seroepidemiological survey was carried out in Southeastern Senegal in order to assess if the most abundant non human primates of the region could potentially act as efficient DENV reservoirs or amplification hosts and play an important role in the virus natural perpetuation in forest galleries where mosquitoes have been found infected with DENV-2. Simultaneously, a YFV serosurvey was conducted.

MATERIALS AND METHODS

The present research complied with legal requirements of the Senegalese authorities and adhered to the principles for the ethical treatment of non-human primates. An authorization to conduct monkey trapping and blood sampling was granted by the Direction of wildlife Services, Ministry of Environment and Nature Protection, Senegal (Approval # 001270 DEF/DGF 2002, Direction des Eaux et Forêts, Chasses et de la Conservation des Sols), and ratified by the Research Institute for Development (IRD, Marseille, France).

Study sites

Ngari village (12° 38' 0.57'' N, 12° 14' 59.77'' W) is located 11 km north of Kedougou in a hilly region of the savanna-forest gallery mosaic of the Sudano-Guinean phytogeographic domain. The rainy season begins in May and ends in October. Ngari, as well as all others surrounding villages, is of traditional agricultural type, consisting of extended family compounds of 3 to 6 houses interspaced between fields of corn, millet and peanuts. Most houses are mud-walled with thatch roofs. Plantations of mango trees (*Mangifera indica*), baobab (*Adansonia digitata*) and *Cola nitida*'s fruits around the village supply a food source for monkeys according to the season. The Pont-Plateau site (12° 36' 0.09'' N, 12° 14' 0.25'' W) is located 2 km south of Ngari in the forest gallery named "PK10" (*i.e.*: 10 km away from Kedougou), bordered by a cool dense forest gallery erected in a depression where mostly baboons and green monkeys sleep. The "Two Rivières" site (12° 38' 0.20'' N, 12° 14' 0.15'' W), located 1 km North of Ngari, represents a temporary running water source bordered by a forest gallery, with high flow during all the rainy season (Figure 1). From May to December 2002, visual surveys were performed in the forest galleries around Kedougou, in order to identify simian species present in the area and to know their vital domains and daily activities. These preliminary studies allowed: 1) to establish the specific richness of monkey population; 2) assess male/female, sub-adult/juvenile ratio for each species. Based on these data, the trapping sites were selected, while also DENV-2 and YFV have been known for circulating in these targeted areas (Cornet et al., 1978; 1979; 1984; Diallo et al., 2003; Traore-Lamizana et al., 1994).

Monkeys trapping and blood collection

Before setting traps, peanut heaps were sparsely placed into rows around the trap places in order to attract monkeys and habituate

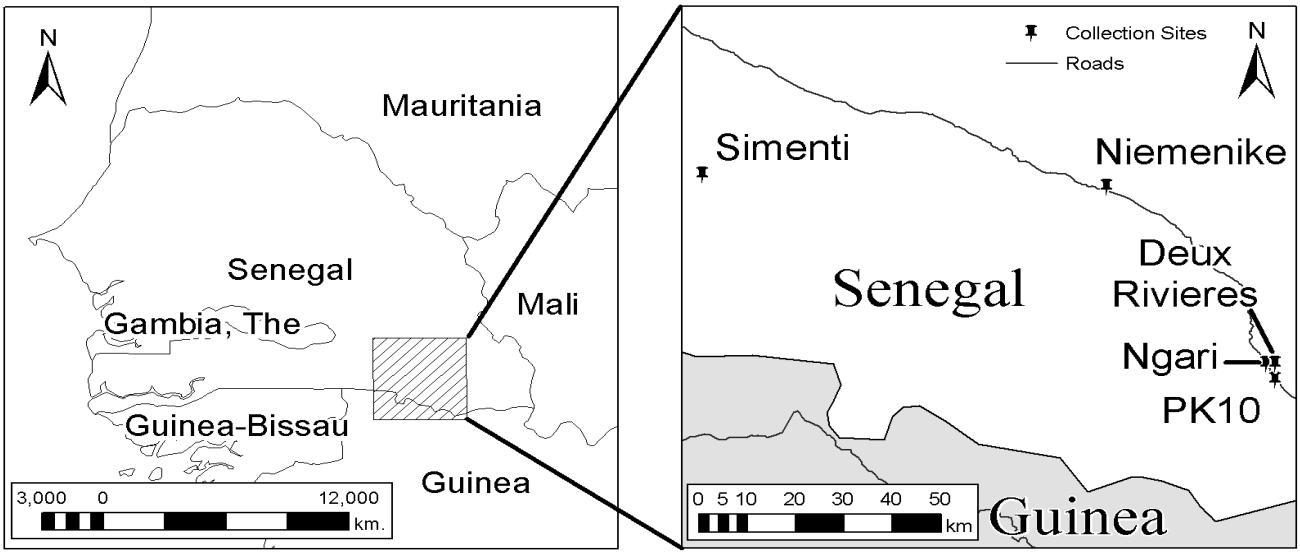

Figure 1. Study sites: Map of Senegal indicating locations of the three trapping sites in southeastern Senegal. Site (N) located about 100 m away from Ngari village; Site (P) for Pont and Site (L) for Plateau are located in the forest gallery of PK10; Site (D) located in Deux Rivieres.

them feeding around the sites. An operator hiding place was set in a small shelter hut under dense vegetation, 150 m distant from each trap to lookout for monkey arrivals. A soft green fishing net was designed for the African green monkey, *Chlorocebus sabaeus* (Gray, 1821) and the Patas monkey, *Erythrocebus patas* (Schreber, 1775) species. It was adapted as a tent trap of 6 m length, 4 m width and 2 m height, maintained vertically by six PVC tubes set at the four corners and two in the middle. Another trap for Guinea baboon, *Papio papio* (Erxleben, 1777) species was made and consisted of a metallic cage of 4 m length and 3 m wide, toughly fixed in the soil by four tubes. Entrance was designed as a sliding door attached to a rope, turning around a pulley, and linked to a tiny rope that ran into the hut for shutter release.

At 06:00 am all material was set ready for capture and blood collection. Trapped specimens were anaesthetized using insulin syringes with a dose of 10 mg/kg of ketamine (Imalgen 1000®). While anesthetized monkeys were taken out of the trap, 5 to 10 ml of blood were drawn from the femoral vein depending to the size of the animal using 10 ml disposable syringes and transferred from the syringe to 10 ml blood sterile collection tubes (VENOJECT® PLAIN SILICON-COATED Z). Samples were stored in a cooler at +4°C to be transported to the research station and processed for sera extraction and preservation. Sera aliquots were kept in Nunc® cryotubes and stored in a nitrogen tank until transferred to a -80°C freezer for later use. Morphometric data were recorded, each individual was weighed and an identification number allocated under his armpit using a dermography stylus.

ELISA test for antibodies detection

YFV and DENV-2 antibody detection were performed on 1/100 sera dilution: IgM were detected by MAC-ELISA following the protocol of Lhuillier and Sarthou (1983) and IgG were detected using the technique of indirect ELISA as previously described (Innis et al., 1989). Serum samples were tested with a positive and negative control. Briefly, specific antibodies bind to soluble antigens attached to the microwells (Titertek, Flow Laboratories, McLean, VA). After a first wash, enzyme conjugate is added to the well that binds antibodies captured by the antigen. After a second wash, a substrate is added that turns blue in the presence of the enzyme complex. A stop solution turns the mixture yellow, and is then read with a spectrophotometer. Results are reported as optical density values (OD).

RESULTS

From June 2002 to December 2006, 58 serum samples were obtained from 51 and seven recaptured, specimens including: 14 *E. patas* and 44 *C. sabaeus* (Table 1).

Among the seven recaptured specimens, three were *C. sabaeus* juvenile males trapped for the first time from Ngari site in December 21st, 2002 (N1, N4 and N6). At their second trapping, on June 3, 2003, their sera were respectively identified as Re1N1, Re1N4 and Re1N6 (Re1N1 meaning 1st Recapture of monkey number N1). While recapturing these individuals at the “Pont” site for a second time, a sub-adult male *E. patas* was captured for the first time and marked as P8 (P for “Pont” site) that same June 3, 2003. Three other juvenile *C. sabaeus* were caught and marked as L1 (female juvenile, L for “Plateau” site), L4 and L10 (both male juvenile) during August, 2006. At that time, the P8 *E. patas* was recaptured (Re1P8, in August 2006). During our last trapping on December 2006, the three *C. sabaeus* previously marked on August 2006 were resampled as Re1L1, Re1L4 and Re1L10.

At the end of the 2002 rainy season, seven sera over 19 of *C. sabaeus* tested positive for YFV IgG, without any YFV IgM detection. Positive individuals were two adult male (D2 and P3), two adult female (D3 and P2) and three juveniles (D4, P5 and N3) (Table 2). Follow up

Table 1. Seroprevalence of YFV and DENV-2 antibodies from trapped monkeys.

Parameter	2002		2003		2006		Total	
	YF	DENV-2	YF	DENV-2	YF	DENV-2	YF	DENV-2
Chlorocebus sabaeus	7/19 (36.8)*	NT	3/9 (33.3)	0/9 (0.0)	NT	4/16 (25.0)	10/28 (3.6)	4/25 (16.0)
Total *C. sabaeus*	19		9		16			44
Erythrocebus patas	0 0	0 0	3/10 (30.0)	0/10 (0.0)	NT	3/4 (75.0)	3/10 (30.0)	3/14 (21.4)
Total *E. patas*	0		10		4		14	
Total monkeys	19		19		20		58	

*Number positive / total tested (Percentage); NT, not tested;

Table 2. Seroprevalence of anti-YFV and anti-DENV-2 antibodies in wild *Chlorocebus sabaeus* and *Erythrocebus patas* captured in Deux rivières (D), Pont (P)-Plateau (L) of PK10, and in Ngari (N) during our study. * (nt = not tested).

Code	Species	Site	Sex	Age	2002				2003				2006			
					YFV		DENV-2		YFV		DENV-2		YFV		DENV-2	
					IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
D2	*C. sabaeus*	2Rivieres	M	Adult	-	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt
D3	*C. sabaeus*	2Rivieres	F	Adult	-	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt
D4	*C. sabaeus*	2Rivieres	F	Juvenile	-	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt
P2	*C. sabaeus*	Pont	F	Adult	-	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt
P3	*C. sabaeus*	Pont	M	Adult	-	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt
P5	*C. sabaeus*	Pont	M	Juvenile	-	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt
N3	*C. sabaeus*	Ngari	M	Juvenile	-	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt
P7	*E. patas*	Pont	M	Subadult	nt	nt	nt	nt	-	+	-	-	nt	nt	nt	nt
P8	*E. patas*	Pont	M	Subadult	nt	nt	nt	nt	-	+	-	-	nt	nt	-	+
P9	*C. sabaeus*	Pont	M	Subadult	nt	nt	nt	nt	-	+	-	-	nt	nt	nt	nt
N7	*E. patas*	Ngari	M	Adult	nt	nt	nt	nt	-	+	-	-	nt	nt	nt	nt
N13	*C. sabaeus*	Ngari	M	Adult	nt	nt	nt	nt	-	+	-	-	nt	nt	nt	nt
N15	*C. sabaeus*	Ngari	F	Juvenile	nt	nt	nt	nt	-	+	-	-	nt	nt	nt	nt
L2	*C. sabaeus*	Plateau	F	Adult	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+
L6	*C. sabaeus*	Plateau	M	Adult	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+
L11	*C. sabaeus*	Plateau	M	Adult	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+
L14	*E. patas*	Plateau	F	Juvenile	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+
L15	*E. patas*	Plateau	F	Adult	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+
L16	*C. sabaeus*	Plateau		Juvenile	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+

studies on the same monkey population during the subsequent 2003 rainy season allowed to test 19 sera from which six were positive for YFV IgG, including three *C. sabaeus* (P9, N13 and N15) and three *E. patas* (P7, P8 and N7). Among them were two adult males [one *C. sabaeus* (N13), one *E. patas* (N7)], three sub-adult males [two *E. patas* (P7 and P8), one *C. sabaeus* (P9)], and one juvenile *C. sabaeus* (N15). No YFV IgM, nor DENV2-IgG or DENV-2 IgM were detected (Table 2). Among the red monkeys (*E. patas*), one sub-adult male (P8) tested positive for YFV IgG on June 2003, and subsequently when recaptured in August 2006, it tested positive for DENV-2 IgG (Table 2).

At the end of the 2002 rainy season, all 19 samples were negative for both DENV-2 IgG and IgM (Tables 1 and 2). During the rainy season in 2006, over 20 sera collected from captured monkeys, seven [four *C. sabaeus* (L2, L6, L11 and L16) and three *E. patas* (Re1P8, L14 and L15)] tested positive for DENV-2 IgG without DENV-2 IgM. Among these, six newly captured individuals in 2006 tested positive for DENV-2 IgG (Table 2), including two juveniles less than 1 year old [one *E. patas* (L14) and one *C. sabaeus* (L16)], attesting that DENV-2 recently circulated within the monkeys of the forest gallery of PK10.

DISCUSSION

YFV IgG positive samples referred to two adult male, two adult female and three juvenile *C. sabaeus* (Table 2). Morphometric and morphologic traits recorded on juveniles allowed for age estimation of approximately two to three years old. Then, one can estimate that these *C. sabaeus* got an YFV infection earlier at the beginning of their life in 1999 and seroconverted that might explain YFV IgG circulation detected in 2002. Another scenario is that, they could have contracted the virus more recently (six months before they were caught and sampled, since YFV IgM disappear within 2 to 5 months). In all cases, YFV reacting antibodies among juvenile not older than 3 years old, in absence of any YF human case reported, attest about a YFV amplification and circulation within monkeys in a silent cycle in the PK10 forest gallery. yellow fever (YF) occurs only in sub-Saharan Africa and the tropical regions of South America, where it is endemic and sporadically epidemic. In Africa, the YF sylvan cycle involves the non-human primate reservoir species (*Chlorocebus spp., Erythrocebus spp.*) and the forest mosquitoes [*Aedes aegypti aegypti, Ae. aegypti formosus, Ae. (Stegomyia) africanus, Ae. (Stegomyia) bromeliae, Ae. (Diceromyia) furcifer, Ae. (Stegomyia) luteocephalus, Ae. (Stegomyia) metallicus, Ae. (Stegomyia) opok, Ae. (Stegomyia) simpsoni complex, Ae. (Diceromyia) taylori, Ae. (Aedimorphus) vittatus*] that bite and infect humans who enter the forest (Cordellier, 1991). The forest savannah mosaic of southeastern Senegal represents the YFV “zone of emergence” where transmission to humans occurs when the fundamental of emergence, including several sylvan and domestic infected mosquito vector species, a preexisting primate-mosquito sylvan YFV cycle and a non immune human population, are combined. The human intrusion in the sylvatic cycle fosters an intermediate YFV cycle that bridges the sylvan enzootic and urban endemic cycles. Ultimately, it is from this scenario that YFV transmission goes from human to human, causing outbreaks and even epidemics affecting several villages and towns in the urban cycle (Germain, 1986).

Moreover, our findings suggest that DENV-2 has been circulating in the PK10 forest gallery of southern Senegal within the local monkey population including *E. patas* as well as *C*. *sabaeus*. DENV-2 isolation in Senegal was first obtained from blood of a young girl in Bandia (14°35"N, 17°01"W; Mbour Department, Thies Region), in the sahelo-sudanian area, in 1970 (Robin et al., 1980). Further entomological investigations conducted in the forest galleries of southeastern Senegal (zone of emergence) led to isolate DENV-2 from *Aedes* (*Stegomyia*) *luteocephalus* mosquitoes in 1974 (Robin et al., 1980). A retrospective non human-primates serosurvey in this area detected also epizootics of DENV-2 infection among monkeys, suggesting that primates might be efficient amplifying hosts for the virus (Saluzzo et al., 1986), and therefore involved in a sylvatic cycle of DENV-2.

Routine entomological surveillance and sero-survey programs set up and carried out by Pasteur Institute and ORSTOM (IRD) of Dakar reported recurrent DENV-2 amplifications in those forest gallery areas of Senegal: 1980-1982, a DENV-2 epizootic occurred with virus isolations from mosquitoes (*Ae. furicifer*, *Ae. taylori* and *Ae.luteocaphalus*) and from the red monkey, *E. patas* (Cornet et al., 1984); 1989-1990, with virus isolation from the same mosquito species as previously found (Traore-Lamizana et al., 1994); 1999, when *Aedes (Stegomyia) aegypti* and *Aedes (Aedimorphus) vittatus* were, for the first time, found infected with DENV-2, while the known potential vectors (*Ae. furcifer, Ae. taylori* and *Ae. luteocephalus)*, were again found infected with DENV-2 and, ultimately DENV-2 IgG were also detected in African green monkeys, *C. sabaeus* (Diallo et al., 2003) captured from January 31 to February 6, 2000 in the same forest galleries (Diallo et al., 2003), as for the present study. Our findings appeared during August of the rainy season of 2006 that is six years after the last DENV-2 amplification of 2000 reported by Diallo et al. (2003), corroborative to the periodicity of occurrence with silent intervals of 5 to 8 years so far observed (Althouse et al., 2012). Moreover, the seroconversion that we have detected from wild *C. sabaeus* and *E. patas* living in forest galleries of southeastern Senegal support the role played by monkeys in the circulation and maintenance of sylvatic DENV-2. After an inter epizootic period, DENV-2 virus reemerged in this area, sharing the same Cercopithecidae vertebrate hosts with YF virus.

Stegomyia mosquitoes (*Ae. aegypti formosus* and *Ae. luteocephalus*) and *Diceromyia* (*Ae. furcifer* and *Ae. taylori*), which are specific to the forest gallery, have been found infected with DENV-2, as well as *Ae. vittatus* (Diallo et al., 2003). They play a major role in the mosquito-monkey maintenance wild cycle regarding their preferences to blood feed on monkeys when they return to the forest gallery at dusk to rest. Also *Ae. furcifer* and *Ae. luteocephalus* were highly susceptible to both sylvatic and urban DENV-2 strains and represent potential vectors of the virus (Diallo et al., 2005). Ultimately, entomological and sero-epidemiological surveillance of arboviruses circulation in Southeastern Senegal (Monlun et al., 1993; Diallo et al., 2003) revealed an amplification of DENV-2 within *Aedes* mosquitoes from the forest galleries, concomitant to DENV-2 infection in humans in the nearby villages (Zeller et al., 1992; Traore-Lamizana et al., 1994).

In other parts of West Africa, Fagbami et al. (1977) detected DENV-2 antibodies in non-human primates inhabiting both gallery and lowland forests in Nigeria; over 100 strains of DENV-2 were also isolated from forest *Ae. taylori, Ae. furcifer, Ae. opok, Ae. luteocephalus* and *Ae. africanus* in Guinea, Côte d’Ivoire, and Burkina Faso (Cordellier et al., 1983; Roche et al., 1983; Hervy et al.,

1984; Rodhain, 1991). In West Africa, there has been no evidence of dengue epidemic from an enzootic transmission that bridge to a rural or urban cycle, affecting human population. Moreover, Rico-Hesse (1990) attributed the epidemic that arose in Burkina Faso in 1982 to a DENV-2 strain that originated from the Seychelles Islands.

In South East Asia, Simmons et al. (1931) conducted some experiments in Manila (Philippines) and prove for the first time that dengue virus can be transmitted by *Aedes* mosquitoes to monkeys species *Macacus fuscatus* and *Macacus philippinensis* and retransferred to other monkeys or to men through mosquito bites. In Penang, Malaya, Smith (1956) demonstrated that forest tree-dwelling mammal species were more exposed to dengue infection than ground-dwelling animals and suggested then, an implication of a canopy-dwelling forest vector. He postulated also that *Ae. albopictus* may be the bridge vector between monkeys in the forest and man in rural areas (Smith, 1958).

Rudnick (1965) demonstrated the presence of widespread DENV-neutralizing antibodies in wild monkeys (*Macaca nemestrina, M. fascicularis, Presbytis cristata* and *P. melaphos*).

Rudnick et al. (1986) isolated several strains of DENV-1, 2 and 4 from 27 sentinel monkeys [*Presbytis obscura* and *Macaca fascicularis* (=*irus*)] placed in the forest canopy while no isolation was obtained from 19 sentinel monkeys placed at ground level. Although DENV-3 has not been isolated, seroconversion in sentinel monkeys suggested their circulation (Rudnick, 1986). They also isolated DENV-2 from *Ae. albopictus,* a potential vector found at ground level in the study areas, and DENV- 4 from an *Aedes* species of the *niveus* group. Furthermore, a serum survey of 300 forest-dwelling Orang Asli aborigines detected neutralizing dengue antibodies in the vast majority, although no clinical dengue was reported among this group (Rudnick, 1986). Based on those findings, they hypothesized that dengue serotypes were circulating in the forest canopy, between *Aedes* mosquitoes of the *niveus* group and monkey species of the genus *Macaca* and *Presbytis* and that the man was occasionally infected by intrusion in this cycle (Rudnick, 1965; Rudnick et al., 1967). Moreover, Yuwono et al. (1984) postulated that this enzootic cycle could occur in all primary forests of tropical Asia where the zoonotic reservoir exists.

This arboviral disease increases its range of occurrence, gaining the tropical and intertropical world because substantial vector control efforts have not stopped its rapid emergence and global spread (Bhatt et al., 2013). DENV epidemics occurred earlier in Zanzibar (Christie, 1881) and in Cairo, Egypt (Hirsch, 1883). Later, it emerged sporadically in Burkina-Faso, in 1925 (Legendre, 1926), in Senegal (Bideau, 1925) and in South Africa (Edington, 1927). After Nigeria epidemic in 1964 diagnosed by a retrospective serosurvey (Carey et al., 1971), the virus spread silently throughout Africa. Kading et al. (2013) recently reported prevalence of antibodies to DENV-2 in non human primates in the greater Congo basin. So far considered as benign without severe syndrome (no dengue hemorrhagic fever) (Gratz and Knudsen, 1996), dengue sporadically emerged in the non immune human population causing hemorrhagic fever and sometimes fatal cases. In fact, an imported DHF case caused by a West African sylvatic strain of DENV-2 in a healthy man returning to Madrid from Guinea Bissau through Senegal has been recently described (Franco et al., 2011). Moreover, an urban epidemic of DEN attributed to serotype 3 occurred in Senegal in 2009, affecting 196 persons with five cases of dengue hemorrhagic fever and one fatal case of dengue shock Syndrome (Faye et al., 2014). A DENV-3 epidemic has also been previously reported in Mozambique (Gubler et al., 1986).

DENV-2 isolates from the above mentioned studies, and isolates from mosquitoes in other parts of West Africa, are phylogenetically distinct from contemporaneous DENV-2 strains circulating in Asia and the Americas, and are therefore likely to constitute a distinct “African” sylvatic cycle (Vasilakis et al., 2012). Recently, a phylogenetic study from Vasilakis et al. (2008) demonstrated that the first dengue virus infection in Nigeria documented by Carey et al. (1971) was an African strain of sylvatic origin. Two distinct transmission cycles have been described for dengue virus: 1) the endemic and epidemic cycles involving human host and viruses are transmitted by main vectors as *Ae. aegypti, Aedes albopictus* and other mosquitoes as secondary vectors (Wang et al., 2000), and 2) the sylvatic natural transmission cycle involving monkeys and several *Aedes spp.* mosquitoes mostly identified in Asia and West Africa (Holmes and Twiddy, 2003).

For a better understanding of the DENV evolution and dissemination throughout Africa, a long term serosurveillance program including non-human primates, and eventually other mammals living in the forested areas, must be undertaken, particularly in West Africa. Moreover, as postulated by Vasilakis et al. (2012), it is possible that sylvatic dengue may be present but yet unrecognized in other regions of Africa.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Antimicrobial potential of *Rothmannia longiflora* Salisb and *Canna indica* Linn extracts against selected strains of fungi and bacteria

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This study was conducted to investigate the antimicrobial activities of the methanolic extract of *Rothmannia longiflora* and *Canna indica* leaves against 10 pathogenic bacterial and fungal strains. The methanolic extract from the two plants were screened for their minimum inhibitory concentration (MIC) against the microbial growth of *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumonia, Candida albicans, Aspergillus niger, Phjapus stolometer* and *Penicillium notatum.* The results show that inhibition of microbial growth decreased with decreasing concentrations of the plant extracts. While concentrations of 200 and 100 mg/ml completely inhibited microbial growth, lower concentrations (50 and 25 mg/ml) showed partial inhibition, extracts at lower concentrations than these had no effects on microbial growth of microorganisms tested. Comparatively, *R. longiflora* gave better results than *C. indica*. This study confirmed the effectiveness of methanolic extracts of the two plants as inhibitory effect to microbial growth of several pathogenic microorganisms and testified to the basis of the ethnomedicinal uses of these two plants against several microbial infections.

Key words: Antimicrobial potential, *Rothmannia longiflora*, *Canna indica*, bacteria, fungi, Nigeria, methanolic extract.

INTRODUCTION

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical inter-mediates and chemical entities for synthetic drugs. The increase of microbial infections have increased dramatically in the past 20 years because of the increase in the number of people whose immune systems are compromised by AIDS, aging, organ transplant and cancer therapy.

Accordingly, increase in the rates of morbidity and mortality because of microbial infections have been regarded as a major problem (Tatli and Akdemir, 2005). Worse still, there are global problems of multiple antibiotics resistance as well as emergence of new and resurrection of previously eradicated diseases. Most of the current antimicrobial drugs simply reduce the level of growth of bacteria or fungi, and some of them are very toxic to the kidney, the hematopoietic and central nervous

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system (Tatli and Akdemir, 2005). Furthermore, antimicrobial resistance among enteric pathogens is becoming a matter of serious concern (El-Mahmoud et al., 2008) and poses a great threat to global human health. Further, new microbial strains are being continuously discovered, which are refractory to the current arsenal of drugs (Erturk et al., 2006). This is because antimicrobial resistance leads to therapeutic failures of empirical therapy (Parekh and Chanda, 2007).

As a result, it has become necessary to fight against emerging and re-emerging infectious diseases with a view to discover and invent new agents of greater therapeutic profile to mitigate frequent outbreaks of diseases which has posed a new threat to global health security (Mohanta et al., 2007). The continuous evolution of bacterial resistance to currently available antibiotics has necessitated the search for novel and effective antimicrobial compounds (Fagbemi et al., 2009), to which these micro-organisms are yet to develop resistance (El-Mahmoud et al., 2008).

With the rising problems of side effects and limited efficacy of antibiotic drugs (Gupta et al., 1998), there is an urgent need for the development of alternative antimicrobial substances and researchers are nowadays turning to natural products from plants (Nitta et al., 2002), as their main source of bioactive compounds with antimicrobial properties, to complement the existing synthetic antimicrobial drugs that are gradually becoming less potent against pathogenic microorganisms (Lawal et al., 2012).

The rate of interest in plant derived drugs has shoot up due to the fact that herbal medicine is safer and less costly than the synthetic drugs which possess serious side effects. It is therefore necessary to screen plants for promising biological activity (Lawal et al., 2010).

Rothmannia longiflora Salisb. is a shrub belonging to the plant family of Rubiaceae, having trumpet-like flower with fruits that are longitudinal in shape. It has been reported to have antimalarial effects and also used in the treatment of measles and to give tribal marks (Ikpi et al., 2009). It is used as chewing stick in the treatment of filariasis, dysentery, and fever and also as an analgesic and emetic. Furthermore, the plants is considered to have febrifugal and analgesic properties, and a decoction of the leaves, twigs, bark and roots is applied internally or externally in lotions, washes and baths. In Nigeria the roots are used to treat bowel complaints (Jansen, 2005).

The plant, *Canna indica* L. is a native of the Caribbean and Tropical America; and belongs to the family Cannaceae. The introduction of this plant to Africa, especially Nigeria is unknown, but it is widely distributed throughout Nigeria, where it is known locally as “ebesalebo” in Edo, “nkwa ebotri” amongst the Efik, “bakalekale” in Hausa, “aberekamwo” in Igbo land and “iroro” amongst the Yorubas. It has been reported to be used ethnomedicinally for the treatment of malaria in South-western Nigeria, as well as a cure for diarrhoea and dysentery and in the treatment of fever, bruises and cut (Odugbemi et al., 2007). Josephine et al., 2013 reported that methanolic extracts of *C. indica* possessed anti-diarrhoeal properties.

This study was therefore conducted to investigate the antimicrobial activities of the methanolic extract of *R. longiflora* and *C. indica* leaves on some bacterial and fungal strains.

MATERIALS AND METHODS

Plant materials

Fresh leaves of *R. longiflora* and *C. indica* were collected from Forestry Research Institute of Nigeria. The plants confirmation and identification was done at Forest Herbarium, Ibadan (FHI) in Forestry Research Institute of Nigeria (FRIN). The leaves were air dried for five days and pulverised using hammer mill and kept for analysis.

Test micro-organisms

A total of 10 test microorganisms used in this study, which include bacterial and fungal strains. The bacteria species used are *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella typhi* and *Klebsiella pneumoniae*; while the fungi species include *Candida albicans, Aspergillus niger, Phjapus stolometer* and *Penicillium notatum*. The micro-organisms were collected from Departments of Pharmaceutical Microbiology and Veterinary Microbiology of the University of Ibadan and the International Institute of Tropical Agriculture (I. I. T. A), Ibadan.

Preparation of the extracts

100 g of the powered plant leaf samples were macerated with 100 ml of 90% methanol for 72 h and pooled to obtain the crude methanol extract. The extract was filtered through filter paper to remove all insoluble matter, including cellular materials.

Antimicrobial assays

4 ml of the sample was measured into the first test tube and five other test tubes contained 2 ml of methanol. From the first tube that contains 4 ml of the original sample, 2 ml was taken into the second tube to make up to 4 ml. This was done until the 6th test which was the last test tube for the extract. The 7th and 8th tubes contain positive and negative controls respectively.

Pour plate method (Bacteria)

An overnight culture of each organism was prepared before taking scoopful of the organism from the stroke and inoculated each into the sterile nutrient broth of 5 ml for 18 - 24 h at 37°C. From overnight culture 0.1 ml of each organism was taken into 9.9 ml of sterile distilled water to get (1:10) 10^{-2} of the dilution of the organism.

From the diluted organism, 0.2 ml was taken into the prepared sterile nutrient agar which was incubated at 45°C. These were then aseptically poured into sterile petri dishes and allowed to solidify for about 45 min. using a sterile cork -borer. The wells were made accordingly into the graded concentrations of the extract including

Table 1. Antibacterial and antifungal activities of methanolic leaf extract of *Rothmannia longiflora* at different concentrations

Test organism	Mean inhibition zone of *R. longiflora* at different concentration						
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	Gmc (10 ug/ml)
Bacteria							
Staphylococcus aureus	17	14	12	10	na	na	38
Isherichia coli	15	12	10	na	na	na	36
Bacillus subtilis	14	10	na	na	na	na	38
Pseudomonas aeruginosa	12	10	na	na	na	na	36
Salmonella typhii	19	14	12	10	na	na	34
Fungi							
Klebsiella pneumoniae	14	10	na	na	na	na	36
Candida albicansna	17	14	12	10	na	na	26
Aspergillus niger	15	12	10	na	na	na	24
Phjapus stolometer	12	10	na	na	na	na	26
Penicillum rotatum	14	12	10	na	na	na	24

Gmc: Gentamycin; NA : Not active.

the controls. The duplicates were made to ascertain the results obtained. The plates were allowed on the bench for about 2 h to allow the extract to diffuse properly into the nutrient agar that is, Pre diffusion. The plates were incubated for 18-24 h at 37°C.

Surface plate method (fungi)

A sterile Sabouraud Dextrose Agar was prepared and aseptically poured into the sterile plate in duplicate and allowed to solidify. 0.2 ml of the 10^{-2} of the organism was then spread on the surface of the agar using a sterile inoculation loop to cover all the surface of the agar. Wells were made using a sterile cork-borer of 8 mm in diameter and into each well, the graded concentration of the extract was put including the controls. All the plates were allowed to stand for 2 h to allow the extract to diffuse properly into the agar i.e. pre diffusion. The plates were incubated for 48 h at 27°C.

Minimum Inhibition Concentration (MIC) test

The method used for determining the minimum inhibitory concentrations (MICs) for the extract followed those of Clinical Laboratory Standardization Institute (CLSI, M100-S18), 2 ml of each concentration to be used was added to 18mls of the agar, and allowed to set. The diluted organisms were streaked on the agar. The plates were incubated for 24 h at 37°C and checked for growth. The MIC was taken as the lowest concentration of the extract that showed no visible growth. This was applied to the controls as well.

RESULTS AND DISCUSSION

The study was conducted to investigate the antimicrobial potentials of the methanolic extracts of *R. longflora* and *C. indica* on 10 selected pathogenic micro-organisms. The antimicrobial potentials of these 2 plants were determined based on the zones of inhibition of microbial growth of the test micro-organisms. The mean zones of inhibition of growth of the isolates are a function of relative antibacterial and anti-fungal activities of the extracts. The zone of inhibition is simply the area on the agar plate that remains free from microbial growth. The size of the zone of inhibition is usually related to the level of antimicrobial activity present in the sample or product - a larger zone of inhibition usually means that the antimicrobial is more potent (Lawal et al., 2012). Some of the plates exhibited clearly distinct zone of inhibition, while some of them did not. Tables 1 and 2 present the calculated mean zones of inhibition (mm) of methanolic leaf extracts of *R. longiflora* and *C. indica* on the bacterial and fungal strains used for this study respectively.

The results show that at the higher concentrations of 200 and 100 mg/ml, methanolic extracts of *R. longiflora* showed inhibition of all the microorganisms (Table 1). The results were similar at the same concentrations in *C. indica*, except for *P. aeruginosa* at 100 mg/ml (Table 2). Inhibition of the methanolic extracts of these plants reduced with decreasing concentrations, with no inhibition recorded at concentrations of 12.5 mg/ml and lower (Tables 1 and 2). Comparatively, *R. longiflora* showed higher inhibition to microbial growth than *C. indica*.

Resistant bacteria have become commonplace in healthcare institutions and increased mortality rate due to resistant *P. aeruginosa, S. aureus, K. pneumoniae, E. coli, Enterobacter* spp., and coagulase-negative staphylococci and enterococci has been reported (Karlosky et al., 2003; NNIS, 2004; Kang et al., 2005). With this increased incidence of antimicrobial resistance and appearance of new infectious for their antimicrobial activity and resistance modifying ability (Gibbon, 2004; Coutinho et al., 2009). While the natural products are known to play significant roles in the development of novel drugs and served as leads for the treatment and

Table 2. Antibacterial and Antifungal activities of methanolic leaf extract of *Canna indica* at different concentrations.

Test organism	Mean inhibition zone of *C. indica* methanol extract at different concentration						
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	Gmc (10 ug/ml)
Bacteria							
Staphylococcus aureus	13	10	na	10	na	na	36
Esherichia coli	13	10	na	na	na	na	38
Bacillus subtilis	13	10	na	na	na	na	36
Pseudomonas aeruginosa	10	na	na	na	na	na	36
Salmonella typhii	14	10	na	na	na	na	34
Fungi							
Klebsiella pneumoniae	16	13	10	na	na	na	32
Candida albicansna	15	13	10	na	na	na	26
Aspergillus niger	14	12	10	na	na	na	24
Phjapus stolometer	16	14	12	10	na	na	29
Penicillum rotatum	15	13	10	na	na	na	28

Gmc, Gentamycin; na, not active.

prevention of diseases (Belini et al., 2008), plant-derived antimicrobials provide the much needed therapeutics (Olajuyigbe and Afolayan, 2013) .

The leaves of *C. indica* showed antimicrobial activity (Abdullah et al., 2012), analgesic activity, and the rhizomes showed a good anthelmintic activity against Pheritima posthuma (Nirmal et al., 2007). The leaves haves chemical constituents like lignin, furfural and hemicelluloses, while rhizomes has 5,8- Henicosdine, Tetracosane and Tricosane (Deming and Tinoi, 2006). The water extract of rhizomes of *C. indica* has been reported to have HIV-1reverse transcriptase inhibitory activity (Woradulayapinij et al., 2005) while its essential oil shows antibacterial activity (Indrayan et al., 2011). Methanolic extract of Aerial Parts of *C. indica* shows antioxidant activity (Joshi et al., 2009). Furthermore, anthocyanins and methylated anthocyanidin glycosides were also isolated from *C. indica* flowers (Srivasta and Vankar, 2010; Srivasta and Vankar, 2010).

Previous studies have shown that *R. longiflora* is the only higher plant that possessed 4-oxonicotinamide-1-(1'-β-D-ribofuranoside), which is only found in human urine. It is an interesting compound, particularly in influencing enzymatic processes. Furthermore, Ebigwai et al., 2012 reported the larvicidal effects and use of *R. longiflora* extracts against *Simulium yahense* larvae. *S. yahense* is responsible for causing onchocerciasis.

Conclusion

This study has confirmed the effectiveness of methanolic extracts of the two plants as inhibitory to microbial growth of several pathogenic microorganisms. Furthermore, the results explain the basis of the ethnomedicinal uses of these two plants against several microbial infections. However, further studies might be required to isolate the active ingredients in the plants.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Myco-epidemiologic and genetic study of dermatophytosis and non-dermatophytes in Middle Euphrates, Iraq

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Dermatophytes are the most prevalent infections in human and animals. These infections can cause disease in all age groups and sexes. The objective of this study was to assess the age and sex prevalence, the site of involvement of different kinds of dermatophytes and an evolutionary study of cross sections type in a private clinic patients attended in Middle Euphrates provinces of Iraq (Babylon, Najaf, Dewania and Karbala) in order to prevent and cure these complications. To survey the dermatophytosis, request forms were prepared to evaluate data like age, sex, profession, site of the lesions, the type of lesions and record of contact with animals. Eighty six clinical specimens from the infected sites were collected from 200 patients (125 males and 75 females) and subjected to conventional methods, 16 isolates were diagnosis by molecular assay, and the results were statistically analyzed by SPSS. The results show that 72.7% of the lesions were dry and 27.3% were inflammatory. The dry lesions were mostly (86%) on the head area and inflammatory ones were mostly (54.7%) in the feet area, 21.4% of the patients had previous contact with animals. Tinea corporis was the most prevalent, while Tinea faciei was the least occurring lesion with the highest cultural isolation rate when compared with *Trichophyton tonsurans, Trichophyton rubrum, Trichophyton violaceum, Epidermophyton, Trichophyton verrucosum* and non dermatophytes. The typing of ITS1-5.8S-ITS2 fragment of rDNA gene for 16 isolated dermatophytes and non-dermatophytes were classified into *Epidermophyton floccosum*, *T. rubrum, Trichophyton mentagrophytes, T. tonsurans* and others molds. The observations of molecular analysis were *T. rubrum*, 800 bp and T*. mentagrophytes*, 690 bp.

Key words: Myco-epidemiologic, dermatophytosis, diagnosis, microscopy, polymerase chain reaction (PCR), Iraq.

INTRODUCTION

Dermatophytes are keratinophylic fungi that are able to infect keratinized tissues of human leading to infections that are mainly restricted to the corneocyte of skin, hair and nails (Adefemi et al., 2011). They fungi belong to three

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anamorphic genera: *Trichophyton, Microsporum* and *Epidermophyton*. (Weitzman and Summerbell, 1995; Adeleke et al., 2008). The infection which is caused by these fungi is termed Dermatophytosis and is commonly referred to as ringworm or tinea. These filamentous fungi are usually identified on the basis of clinical features and isolation patterns together with conidial shapes and sometimes with physiological characters such as; hair perforation and urease tests. In some cases, morphological identification can be difficult or uncertain because there is considerable variation among isolates of some species, and it is a time-consuming procedure requiring even 30 day for final isolation and identification of etiologic agent at genus or species level. Likewise, in some instance, the causative dermatophyte fail to produce any obvious reproductive structure in culture (termed sterile mycelia) which makes it impossible for ultimate definitive diagnosis. On the other hand, antifungal drugs are expensive, and they have many side effects in humans and animals. In the last decades, genotyping approaches have proven to be useful for solving problems of dermatophyte taxonomy, as well as enhancing the reliability and speed of dermatophytosis diagnosis (Aho, 1980; Faggi et al., 2001; Jousson et al., 2000; Mochizuki et al., 2003; Jousson et al., 2006; Graser et al., 2006).

Genotyping method offers an alternative way of identifying individual fungal isolates for epidemiological purpose. Genotyping procedure had previously been developed based upon variable rDNA gene cluster of *Trichophyton rubrum* (Jackson et al., 2000). Recently, *Trichophyton mentagrophytes* var. *interdigitale* has also been shown to possess genetic polymorphisms that map to the rDNA (Mochizuki et al., 2003). By development of PCR technology, a wide variety of molecular techniques such as RAPD-PCR, nested-PCR, PCR-RFLP, PCR-EIA, Real-time PCR and microarray technology were employed as possible alternatives for routine identification of fungi including dermatophytes (De Baere et al., 2010).

Considering the financial and physical costs that dermatophytosis infections impose on the society, urgent actions must be taken to prevent the disease and cure it.

The objective of this survey was to assess the age and sex prevalence, the site of involvement of different kinds of dermatophytes in order to prevent and cure the complication. The ITS1-5.8S-ITS2 fragment of ribosomal DNA gene (rDNA) in the dermatophyte species were used as a reliable marker for species identification.

MATERIALS AND METHODS

This survey which is an evaluating study, was carried out on two hundred patients infected with dermatophytosis, their age ranged from 1-70 years (mean 19.8±12.5 SD) they visited and were referred to dermatology private clinic in Hilla (in which patients come from Hilla, Karbala, Dewania and Najaf provinces, all these provinces are called Middle Euphrates). This study was conducted in from March 2012 to March 2013.

The characteristics of the patient such as age, sex, profession, address, the record of contact with animals, the site of the lesion, the type of lesion (dry or inflammatory) were collected in advance forms.

Hair and skin

Specimens from the infection sites were collected and subjected to conventional examining by directed (potassium hydroxide) KOH. Microscopic examination and 85 specimens were cultured on primary and selected media. Dermatophytes isolates were identified by their colony characteristics: microscopy, physiologic and biochemical test .16 specimens were genotyped by PCR.

Extraction of DNA

Sixteen fungi isolated were used for DNA extraction and PCR assay. DNA of fungal isolates were extracted by picking 1 g of mycelia by using sterile loop and suspending into 300 µl of lysis buffer (10 mM Tris, 1mM EDTA (pH8), 1% SDS, 100 mM NaCl, 300 µl phenol-chloroform (1:1)) shaken for 5 min and centrifuged at 1000 rpm, the supernatant was transferred to new tube and equal volume of chloroform was added, mixed, centrifuged and the supernatant was transferred to new tube. 500 µl of 70% ethanol alcohol was mixed with supernatant and centrifuged at 10000 rpm for 7 min, dry DNA pellet was re-suspended in 75 µl of TE buffer and stored at - 20°C until use (Mousavi et al., 2007).

PCR assay

The ITS1-5.8S-ITS2 region of rDNA from various reference strains of fungal species (dermatophytes and non-dermatophytes) were amplified using the universal fungal primers ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) used to amplify the entire ITS rDNA region in dermatophytic fungi and non-dermatophytic fungi. The PCR mixture was amplified by thermal cycler PCR System (Labnet, USA) using the following conditions: first denaturized temperature of 95°C for 5 min, followed by 30 cycles including initial denaturation temperature of 95°C for 30 s, annealing temperature of 57°C for 1 min, extensions temperature of 72°C for 1 min, and final extension temperature of 72°C for 7 min. The PCR products were run on 1.2% agarose gel (Bio Basic Canada Inc.) electrophoreses performed at 100 V in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by Gel imager scope 21 ultraviolet transilluminator (Korea Company).

The Chi-square test was used to find out if there was any relationship between human contacts with animals and the occurrence of tinea.

RESULTS AND DISCUSSION

Two hundred patients were examined, all of them were studied through critical observation, and culture was carried out from 86 patients. From the ages point of view, the patients were at the age group of 1-70 years, the most

Table 1. Concentration, distribution and prevalence, percentage of dermatophyte and non dermatophyte fungi, in terms of the type of the disease in patients that visited private clinic of dermatology (specimens were collected from patients from Hilla, Karbala, Dewania and Najaf province in Iraq).

Type of fungal dermatophytes	Tinea corporis	Tinea capitis	Tinea faciei	Tinea cruris	Tinea pedis	Total of specimens	%
T. mentagrophytes	16	2	2	6	2	28	32.5
T.tonsurans	12	4	0	0	0	16	18. 6
T. rubrum	5	0	0	3	2	10	11.6
T.violaceum	0	9	0	0	0	9	10.46
E. floccosum	0	0	0	0	11	11	12.79
T.verrucosun	0	0	3	0	0	3	3.48
Non-dermatophytes	1	0	0	1	1	3	3.48
Negative cultures	-	-	-	-	-	6	6.97
Total						86	100%

involved age group was 11-20 years which were 73 patients (36.5%), these results were consistent with the results of Oyeka and Okoli (2003) and Ameen (2010). From sex point of view, 125 (62.5%) of the patients were males and 75 (37.5%) females. The males showed the most prevalent infections of tinea corporis 78.7%, tinea cruris 77.5%, and tinea pedis 56.3%. In the females, the most prevalent dermatophyte infections were, tinea manuum 49.3% tinea corporis 48%, and tinea cruris 45.3%. Our results were in concordance with the results of Leite et al. (2014) and Havlickova et al. (2008).

From the lesions site of view, patients had skin lesion 30% in trunk, 25% in lower limb, 6%, in upper limb, 3% in the neck, 10% face, 12% head and 14% groin. From lesion type point of view, there were 72.7% dry and 27.3% inflammatory. The dry lesions were mostly on the head area, 86% and inflammatory ones were mostly in the feet area (54.7%). Statistical study of the patients infected with dermatophytes showed that there was a meaningful relation between the type of profession and the lesion sites (Leite et al., 2014). In some specific conditions, tinea corporis in student and housekeepers (p=0.01), tinea capitis in students and children (p=0.03), tinea cruris in drivers and in staff tinea manuum in housekeepers and farmers and tinea pedis in athletes, staff and free professions were more than other cases (p=0.000) (Malinovsch et al., 2009).

The results of culture in 85 patients showed that *T. mentagrophytes* 28(32.9%) was the most commonly isolated followed by *T. tonsurans* 16(13.8%) and *T. rubrum*, 10 (11.7%) (Table 1).

From the residence point of view, 65 (32.5%) patient were in the villages and 135 (67.5%) patients were resident in cities. There was significant relationship (pv=0.000) between human contact with animals and the occurrence of tinea.

According to obtained statistics on quantity of the lesion, 60.8% of patients had one lesion, 31% of them had two lesions, 5.3% had three lesions, 2.9% had four lesions and totally 37.1% of the patients had more than one lesion. Our results are coincidence with results of Adeleke et al. (2008).

The 77 isolated dermatophyte strains were found to belong to six species: *E. floccosum* (11 isolates) *T. mentagrophytes* (28 isolates), *T. rubrum* (10 isolates), *T. violaceum* (9 isolates), *T. tonsurans* (16 isolates), *T. verrocosum* (three isolates) and 3 isolates of non-dermophytes fungi (Table 1).

Cultural and microscopic features:

Most of fungal isolates showed standard colonies and microscopic characters especially colony colors and conidial shapes (Figures 1, 2 and 3).

Genotyping of fungal isolates

Currently, molecular studies are crucial and necessary for identification (Malinovschi et al., 2009). The amplified ITS1-5.8S-ITS2 fragment (including primers) ranked between 380 bp for *Geotrichium* sp. and 780 bp for *E. floccosum* in amplicon length (Table 2 and Figure 4). These results agree with results of (Rezaei-Matehkolaei et al., 2012). Shehata et al. (2008) showed in their study the variation in amplicon length of ITS1-5.8S-ITS2 in *T. rubrum* 800 bp and T*. mentagrophytes* 690 bp, ribosomal DNA. Some species revealed closely related amplicon length as in lanes 1-4, 7-8 11, (14 approximately 800 bp). The variation in amplicon length for closely related *Trichophyton* isolates may arises from isolates source or some isolate are probably not typical strain of *T. rubrum* or it can be an intermediate strain between *T. rubrum* and

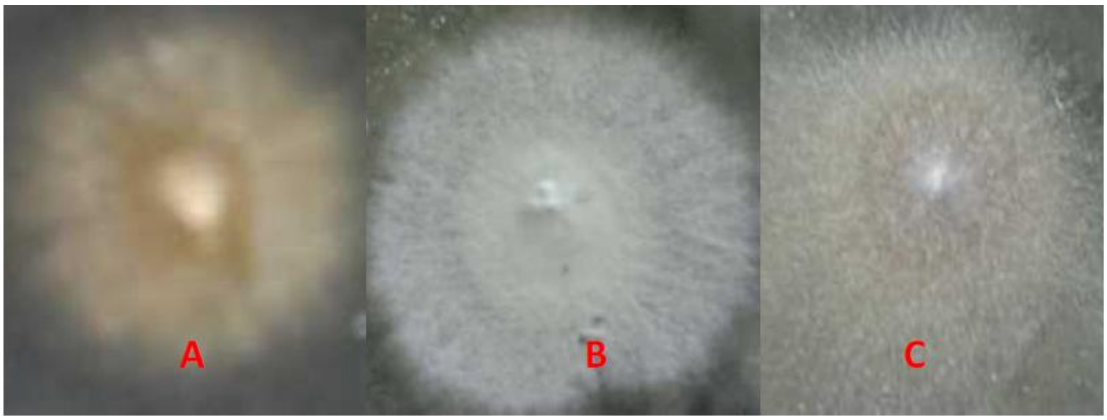

Figure 1. Colonies of A = *E.floccosum,* B = *T. rubrum,* C = *T. mentagrophyte* on Sabouraud`s Dextrose Agar after 14 days incubation at 37°C.

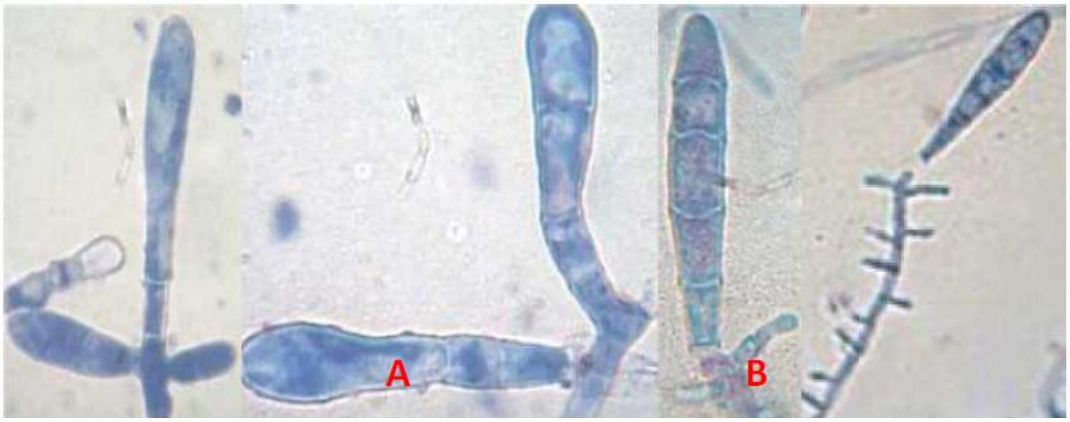

Figure 2. Conidial shape of A = *E. floccosum,* B = *T. rubrum*on Sabouraud`s dextrose agar after 14 days incubation at 37°C.

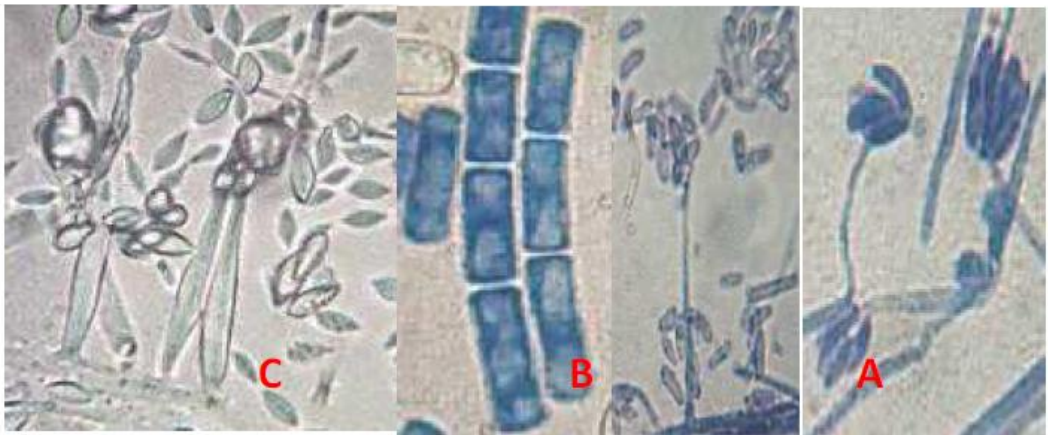

Figure 3. Conidial shape of: A- *Acremonium,* B-, C- *Cladophialophora bantiana* on Sabouraud`s dextrose agar 14 day incubation at 37°C.

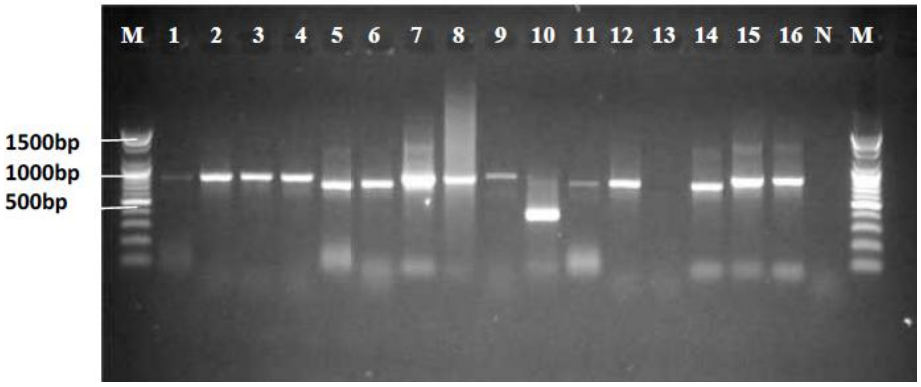

Figure 4. Gel electrophoresis of PCR products of ITS1-5.8S-ITS2 region of rDNA from various isolates of dermatophyte and non-dermatophyte species were amplified using the universal fungal primers ITS1 and ITS4. Lanes: 1-3, 15-16 = *E. floccosum*; 7, 9, 14 = *T. rubrum;* 6, 11-12 = *T. mentagrophytes*; 5 = *T. tonsurans*; 10 =*Geotrichum*; 13 = *Aspergillus flavus*; N = Negative control; M = molecular marker 100 bp.

Table 2. Amplicon length of entire ITS1-5.8S-ITS2 region of dermatophytic and non dermatophytic fungi. Lane M = molecular marker (100 bp). Lanes 1-16 fungal isolates, N = negative control.

Fungal species	Lane number	Amplicon length (bp)
Epidermophyton floccosum	1	780
E. floccosum	2	780
E. floccosum	3	780
E. floccosum	4	780
T . tonsurans	5	680
T. mentagrophytes	6	690
T. rubrum	7	800
E. floccosum	8	780
T. rubrum	9	800
Geotrichum	10	380
T. mentagrophytes	11	720
T. mentagrophytes	12	720
Aspergillus flavus	13	590
T.rubrum	14	720
E. floccosum	15	780
E. floccosum	16	780
Negative control	17	

T. mentagrophytes, these results were consistent with the report of Mirzahoseini et al. (2009). *T. tonsurans* is an anthropophilic source that was isolated only from human infections while others were a zoophilic source (Jackson et al., 2000, 2006; Mirzahoseini et al., 2009).

Conclusion

With regard to the obtained results, the age, sex, the patient contact record with animal and profession has correlation with the occurrence of different types of tinea. The genotypic differentiation by PCR provides a rapid and practical tool for identification of dermatophytes isolates to the species and strain level within one day which is independent of the culture variations.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Inhibitory activities of *Ceiba pentandra* (L.) Gaertn. and *Cordia sebestena* Linn. on selected rapidly growing mycobacteria

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The plants *Ceiba pentandra* (L.) Gaertn. [Malvaceae previously Bombacaceae] and *Cordia sebestena* Linn. [Boraginaceae] used in this study were selected to investigate and justify their local use in the treatment of cough, catarrh, sore throat, pneumonia and tuberculosis, all of which are associated with respiratory tract infections. The antimycobacterial activities of these plants were investigated in *Mycobacterium fortuitum* ATCC 684, *Mycobacterium smegmatis* ATCC 19420, *Mycobacterium abscessus* and *Mycobacterium phlei* ATCC 19240. The agar cup diffusion method was used for the antimycobacterial screening at concentrations of 10, 20, 100 and 200 mg/ml while the agar dilution method was used for the determination of the minimum inhibitory concentration (MIC). Phytochemical screening revealed the presence of tannins, cardenolides, alkaloids, anthraquinones and saponins in all the plant samples except in *C. sebestena* leaf in which saponins and anthraquinones were absent. The inhibitory activity of methanolic extracts of the stem barks of *C. pentandra* and *C. sebestena* on the test organisms was dose-dependent. The MIC and the minimum bactericidal concentration (MBC) of the extracts ranged from 20 - 200 and 40 - 600 mg/ml, respectively. The results obtained in this study justify the ethnomedicinal use of the plants in conditions associated with respiratory tract infections.

Key words: Inhibitory activities, *Ceiba pentandra* (L.) Gaertn., *Cordia sebestena* Linn., rapidly growing mycobacteria, *in vitro.*

INTRODUCTION

Ceiba is the name of a genus of about 17 species in the family Malvaceae, native to Mexico, Central America and the Caribbean, northern South America and tropical West Africa (Gibbs and Semir, 2003). The best-known, and most widely cultivated, species is Kapok, *Ceiba pentandra*. Other species of *Ceiba* include *Ceiba aesculifolia, Ceiba trichistandra, Ceiba chodati* and *Ceiba speciosa*. In the Kano area of Northern Nigeria, they are

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pounded to a fine state to apply as a curative dressing on sores. Pounded leaves are applied as a dressing on sores, tumours, abscesses and whitlows (Burkill and Dalziel, 1997). Leaf infusion is taken orally against cough and hoarse throat. Stem and bark are used as antibacterial, heart tonic and in kidney stone, headache and snake bite. In Nigeria, the bark is used on skin infections, and the bark infusion is taken as a febrifuge (Burkill, 2000). The root is used in various remedies for leprosy in Ivory Coast-Upper Volta (Burkill and Dalziel, 1997). Young leaves are a source of calcium and iron. Seeds contain oil, 24.2% ash, 5.22% crude fiber, 23.9% albuminoids, 18.9% carbos and others, 15.9%. The oil is a mixture of fatty acid, 70% liquid, 30% solid palmitic acid. It also contains fatty acids glycosides, saponins and steroids (Sarkiyayi et al., 2009). Chemicals identified in the floss are pentosans and uronic anhydrides (Burkill and Dalziel, 1997).

Cordia is a genus of shrubs and trees in the borage family Boraginaceae. About 300 species have been identified worldwide, mostly in warmer regions. Many of the species are commonly called manjack while bocote may refer to several Central American species in Spanish (Quattrocchi, 2000). The genus *Cordia* is a known source of benzoquinones, naphthoquinones, hydroquinones, cromenes, triterpenes, sesquiterpenes, polyphenols and flavonoids. Many compounds originally isolated from *Cordia* species have been reported as presenting several biological activities such as antifungal, larvicidal, anti-inflammatory and anti-androgenic (Renata et al., 2005). Syrup of the bark, flowers, or fruit is taken for coughs and bronchial ailments. The tree's sap is applied to wounds. Four phenylpropanoid esters, sebestenoids A-D have been isolated from the fruit of *Cordia sebestena* (Dai et al., 2010). The chemical compounds: robinin, rutin, datiscoside, hesperidin, dehydrorobinetin, chlorogenic acid and caffeic acid have been isolated from *Cordia francisci, Cordia myxa* and *Cordia serratifolia* (Wiart, 2006). The aim of this study is to evaluate and justify the folklore use of *C. sebestena* (L.) Gaertn. and *C. pentandra* Linn. in the treatment of coughs and bronchial ailments associated with lower respiratory tract infections. Rapidly growing mycobacteria similar genotypically but more resistant than *Mycobacterium tuberculosis* were used in this study to evaluate the antimycobacterial potentials of the test plants because they are less pathogenic than *M. tuberculosis* which is actually the main target for developing new antimycobacterial agents.

MATERIALS AND METHODS

Plant collection and identification

Fresh stem bark and leaves of *C. pentandra* (L.) Gaertn. and *C. sebestena* Linn. were collected from University of Ibadan, Oyo State, Nigeria. The plants *C. pentandra* and *C. sebestena* were identified by Mr. Donaltus, a herbarium officer of the Department of Botany, University of Ibadan. The plants *C. pentandra* and *C. sebestena* were authenticated at Forestry Research Institute of Nigeria (FRIN), Jericho, Ibadan, Nigeria and assigned Voucher specimen numbers FHI 108935 and FHI 108934, respectively. The plant parts were air-dried, pulverized, weighed and stored for the study.

Phytochemical screening

The leaf and stem bark of *C. pentandra* and *C. sebestena* were screened for the presence of secondary metabolites such as alkaloids, cardenolides, saponins, tannins and anthraquinone glycosides using the methods described by Harborne (1998).

Extraction procedure and preparation of extracts

Extracts of the test plant samples were obtained by cold extraction at room temperature. Pulverized *C. pentandra* leaves (304.8 g), *C. pentandra* stem bark (1105.7 g), *C. sebestena* leaves (288.1 g) and *C. sebestena* stem bark (661.9 g) were soaked in *n*-hexane for 48-72 h after which the solvent was decanted. The samples were air-dried and soaked in methanol for about 48 h with constant agitation after which the solvent was decanted and more solvent added successively to ensure complete extraction. The methanol extract was concentrated *in vacuo*, weighed and stored for further studies. The crude methanol extracts was partitioned into dichloromethane. The methanol and dichloromethane fractions were collected, concentrated, dried and weighed. Four different concentrations were prepared for each of the crude extract used, and they were 10, 20, 100 and 200 mg/mL. Extracts were reconstituted in 20% dimethylsulfoxide (DMSO).

Antimicrobial screening of plant materials

All the test microorganisms were investigated for their susceptibilities to the various plant extracts by means of agar diffusion technique (Hugo and Russel,1998; Adeniyi et al., 2006, Lawal et al., 2011). The following microorganisms were used in the study: *Mycobacterium fortuitum* ATCC 684*, Mycobacterium smegmatis* ATCC 19420*, Mycobacterium abscessus, and Mycobacterium phlei ATCC* 19240. Overnight bacterial cultures in Tryptic soy broth (Oxoid, UK) were obtained by subculturing from the stored slopes. A 1 in 100 overnight broth culture of each bacterium in appropriate broth medium was made by adding 0.1 ml of the broth into a 9.9 ml of sterile distilled water. Using a sterile pipette, 0.2 ml of the 1 in 100 dilution overnight culture of the test organism was seeded into 20 ml of melted and cooled (45°C) Mueller-Hinton agar (Oxoid, UK) supplemented with 10% blood, poured into sterile Petri-dishes after thorough mixing between palms and allowed to set. The surfaces of the plates were dried in an already disinfected oven at 37°C in an inverted position. Equidistant wells were bored into the solidified medium using a sterilized cork borer of diameter 8 mm. One hundred microlitre (100 µl) volumes of each reconstituted extract at concentrations 10, 20, 100 and 200 mg/ml were introduced into the wells. 10 and 20 µg/ml concentrations of Rifampicin were introduced into two of the wells to serve as positive control while DMSO (20%) was used as the negative control. The plates were left

Table 1. Yield and macroscopic characteristics of extracts of *Ceiba pentandra* (L.) Gaertn. and *Cordia sebestena* Linn.

Plant	Plant part	Weight of ground sample (g)	Yield (g)		Yield (%)		Macroscopic characteristic	
			MeOH	Dichloro.	MeOH	Dichloro.	MeOH	Dichloro.
Ceibapentandra	Leaf	304.8	8.3	4.0	2.7	1.3	Shiny dark-brown congealed extract	Greyish congealed extract
	Stem bark	1105.7	12.1	2.2	1.1	0.2	Shiny reddish-brown and slightly powdery extract.	Dark-brown congealed extract
Cordiasebestena	Leaf	288.1	26.7	7.6	9.3	2.7	Dark-brown congealed extract	Greyish congealed extract
	Stem bark	661.9	29.4	2.8	4.4	0.4	Shiny dark-brown congealed extract.	Shiny reddish-brown extract.

MeOH- Methanol; Dichloro- dichloromethane.

for one hour at room temperature to allow pre-diffusion of the extracts and controls in the well through the agar after which they were incubated at 37°C for 24 h. After incubation, the zones of diameter of inhibition were measured and recorded. All tests were performed in duplicates.

Determination of minimum inhibitory concentration (MIC)

The MIC for the bioactive extracts was determined by the agar dilution procedure guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2008). Different concentrations of the extracts were prepared to final concentration in the range of 200 to 25 mg/ml in 20% DMSO. One millilitre (1 ml) of the extract from each dilution was mixed with 19 ml of melted and cooled (45°C) in Mueller-Hinton agar (Oxoid, UK) was supplemented with 10% blood and poured into sterile Petri dishes allowing the agar to set. The surface of the agar was allowed to dry before streaking with overnight broth cultures of test organisms. The plates were incubated at 37°C for 24 h and examined for the presence or absence of growth. The lowest concentration preventing visible growth was taken as the MIC of the extract. All procedure was performed in duplicates.

Determination of minimum bactericidal concentration (MBC)

The MBC for the bioactive extracts were determined by a modification of the method of Aibinu et al. (2007). Concentrations higher than and equivalent to the MIC were prepared in Tryptic Soy broth (Oxoid, UK), 0.5 mL of a 24 h culture of test organisms were added to 4.5 ml of the extracts solution in test tube. The mixture was incubated at 37°C for 24 h after which aliquots of samples were withdrawn. Ten-fold dilutions were made and 0.2 mL of 10^{-3} dilution was transferred onto extract-free sterile melted and cooled (45°C) in Mueller-Hinton agar (Oxoid, UK) supplemented with 10% blood in Petri dish. The agar plates were incubated at 37°C for 24 h and observed for absence or presence of growth. The minimum concentration preventing visible growth of the organisms was taken as MBC. All procedure was performed in duplicates.

RESULTS AND DISCUSSION

The extraction yield, percentage yield and macroscopic characteristics of the plants extracts are presented in Table 1. The yield in percentage of the methanol extracts of the plant samples was about 5 times more as compared to the dichloromethane extract. This, however, is not in agreement with the report of Cowan (1999) that ranked methanol second next to methylene dichloride (dichloromethane) in terms of yield in extraction of plant active components. This may be due to the fact that the dichloromethane extract was obtained from partitioning of the methanol extracts. The higher yield values of the methanol extracts may suggest that polar compounds abound in the plant samples since methanol is a more polar solvent relative to dichloromethane. Phytochemical screening of the plant samples revealed the presence of tannins, cardenolides and alkaloids in all the plant samples while anthraquinones and saponins were present in all samples except *C. sebestena* leaf (Table 2). These phytochemical compounds are known to be biologically active and thus aid the antimicrobial activities of *C. pentandra* and *C. sebestena.* Phytochemicals exert antimicrobial activity through different mechanisms; tannins for example, act by iron deprivation, hydrogen bonding or specific interactions with vital proteins such as enzymes (Scalbert, 1991) in microbial cells. Tannins are known to provide the typical tanning effect which is important for the treatment of inflamed or ulcerative tissues (Parekh and Chanda, 2007). Herbs that have tannins as their component are astringent in nature and are used for treating intestinal disorders such as diarrhoea and dysentery (Dharmananda, 2003) thus

Table 2. Phytochemical screening of *Ceiba pentandra* (L.) Gaertn. and *Cordia sebestena* Linn.

Phytochemical grouping	Plant/morphological parts			
	***Ceiba* leaf**	***Ceiba* stem bark**	***Cordia* leaf**	***Cordia* stem bark**
Anthraquinones	+	+	-	+
Tannins	+	+	+	+
Saponins	+	+	-	+
Cardenolides	++	++	+	++
Alkaloids	+++	+++	+	+

+ = Low concentration; ++ = moderate concentration; +++ = high concentration.

Table 3. Antimycobacterial screening of *Ceiba pentandra* (L.) Gaertn leaf extracts. Mean diameter of zone of inhibition (mm) ± SEM.

Extract (mg/mL)	Methanol (mg/mL)				Dichloromethane (mg/mL)				RMP (µg/mL)		DMSO
	10	20	100	200	10	20	100	200	10	20	(20%)
M. fortuitum ATCC 684	-	-	12±0.0	12±0.0	-	-	10±0.0	11±0.0	25±0.0	27±0.0	-
M. smegmatis ATCC 19420	-	-	12±0.0	14.5±0.5	-	10±1.5	12±0.0	12±0.0	28±0.0	29±0.5	-
M. abscessus	-	-	-	-	-	-	-	-	20±0.0	20±0.0	-
M. phlei ATCC 19240	-	-	-	-	-	-	-	-	20±0.0	22±0.5	-

Diameter of cork borer = 8 mm, - = resistance, RMP = Rifampicin.

exhibiting antimicrobial activity. The presence of tannins in *C. pentandra* and *C. sebestena* supports the traditional medicinal use of these plants in the treatment of different ailments. Li et al. (2003) reviewed the biological activities of tannins and observed that tannins have remarkable activity in cancer prevention and anticancer, thus suggesting that the plants have potentials as a source of important bioactive molecules for the treatment and prevention of cancer. Moreover, ethnobotany of *C. pentandra* claims that its pounded leaves are applied as a dressing on tumours (Burkill and Dalziel, 1997). In addition to its antimicrobial and anticancer activities, tannins have roles such as stable and potent antioxidants. Tannins have been reported to prevent the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable for them (Sodipo et al., 1991). Alkaloids are compounds needed for cell activity and gene code in the genotype. They are biologically significant as active stimulator and terminators of growth, a part of endogenous security and mechanism. They display antimicrobial and anti-parasitic properties; they are non toxic to the plants that produce them. Biotoxicity is directed only towards foreign organisms or cells and it is selective. Alkaloids can alter DNA and selectively deform cells (Carl, 2007). These biological activities of alkaloids may also have contributed to the the antimycobacterial activities of *C. pentandra* and *C. sebestena.*

The results of the antimicrobial screening of the extracts are presented in Tables 3 to 6 which showed that the methanol extracts of both plants were the most active against the test microorganisms. The activity demonstrated by the fractions was dose-dependent as shown by the diameter of zone of inhibition for most of the active extracts. The diameter of the zone of inhibition for most of the extracts increased as the concentration of the extracts increased. Antimycobacterial screening showed that the test organisms had varied susceptibilities to the extracts, the most susceptible being *M. fortuitum* ATCC 684 while the most resistant was *M. abscessus* (Tables 3 to 6). The most active of the extracts against all the test organisms was *C. pentandra* stem bark. The test organisms also demonstrated varied susceptibility to the control drugs as shown in Tables 3 to 6. From the results obtained in this study, it was observed that the plant extracts demonstrated antimicrobial activities against the selected organisms. This was evidenced by the varying zones of inhibition of the individual extracts on the test organisms. However, 10 mg/ml of the methanol and the dichloromethane leaf extracts of the two plants showed no activity against all the test organisms. The results of the MIC and MBC of the bioactive extracts are shown in Table 7. The MIC value for the bioactive extracts against the bacteria was 20 - 200 mg/ml. *C. pentandra* stem bark methanol extract produced the lowest MIC value of 20 mg/ml for some of the bacteria. This suggests that this

Table 4. Antimycobacterial screening of *Ceiba pentandra* (L.) Gaertn. stem bark extracts. Mean diameter of zone of inhibition (mm) ± SEM.

Extract/control	Methanol (mg/mL)				Dichloromethane (mg/mL)				RMP (µg/mL)		DMSO
	10	20	100	200	10	20	100	200	10	20	20%
M. fortuitum ATCC 684	-	10±2.0	13.5±1.5	15±1.0	-	-	-	11±0.0	25±0.0	27±0.0	-
M. smegmatis ATCC 19420	11±0.0	11±1.0	14.5±1.5	17±1.0	-	-	-	-	28±0.0	29.5±0.5	-
M. abscessus	-	9.5±0.5	10.5±1.5	11±1.0	-	-	-	-	20±0.0	20±0.0	-
M. phlei ATCC 19240	12±2.5	13±3.0	13±0.0	14.5±0.0	-	-	-	-	20±0.0	22.5±0.5	-

Diameter of cork borer = 8mm, - = resistance, RMP = Rifampicin.

Table 5. Antimycobacterial screening of C*ordia sebestena* Linn. leaf extracts. Mean diameter of zone of inhibition (mm) ± SEM.

Extract/control	Methanol (mg/mL)				Dichloromethane		(mg/mL)		RMP (µg/mL)		DMSO
	10	20	100	200	10	20	100	200	10	20	20%
M. fortuitum ATCC 684	-	-	18±0.0	20±0.0	-	-	-	-	25±0.0	27±0.0	-
M. smegmatis ATCC 19420	-	9±0.0	16.5±0.5	17.5±1.5	-	-	-	-	28±0.0	29.5±0.5	-
M. abscessus	-	-	-	-	-	-	-	-	20±0.0	20±0.0	-
M. phlei ATCC 19240	-	-	-	-	-	-	-	-	20±0.0	22.5±0.5	-

Diameter of cork borer = 8 mm, - = resistance, ND = Not determined, RMP = Rifampicin.

Table 6. Antimycobacterial screening of C*ordia sebestena* Linn. stem bark extracts. Mean diameter of zone of inhibition (mm) ± SEM.

Extract/control	Methanol (mg/mL)				Dichloromethane (mg/mL)				RMP (µg/mL)		DMSO
	10	20	100	200	10	20	100	200	10	20	20%
M. fortuitum ATCC 684	-	-	20±0.0	23±0.0	-	-	14±0.0	18±0.0	25±0.0	27±0.0	-
M. smegmatis ATCC 19420	12.5±0.5	12.5±0.5	20.5±1.5	20±0.0	-	11±0.0	15±0.0	19±1.0	28±0.0	29.5±0.5	-
M. abscessus	-	-	-	-	-	-	-	-	22±0.0	20±0.0	-
M. phlei ATCC 19240	-	-	10±0.0	13±0.0	-	-	-	-	20±0.0	22.5±0.5	-

Diameter of cork borer = 8 mm, - = resistance, RMP = Rifampicin.

Table 7. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of bioactive extracts in mg/mL.

Extract/control	Ceiba stem bark MeOH		Cordia leaf MeOH		Cordia stem bark MeOH		Rifampicin (µg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
M. fortuitum ATCC 684	20	40	200	400	200	400	40	80
M. smegmatis ATCC 19420	20	40	200	600	200	400	40	80
M. abscessus	200	600	R	R	R	R	40	80
M. phlei ATCC 19240	200	600	R	R	100	400	40	80

R= Resistance, ND = not determined.

extract has the highest potency, that is, lower dose of this extract will be required to treat or prevent infections when compared with the amount or dose of other extracts that will treat or prevent infections with the same organisms. The MIC of the methanol extracts of the stem barks of *C. pentandra* and *C. sebestena* and the methanol extract of the leaf of *C. sebestena* for all the tested organisms showed varying concentrations. This gives a clue to effective pharmacologic concentrations of the extracts that will be required to treat infections caused by the

test organisms. Generally, the methanol extracts of the morphological parts of the two different plants had better activity when compared with the dichloromethane extracts. The dichloromethane extracts of some of the plant parts demonstrated no antimicrobial activity at all test concentrations. The antimycobacterial activities of the test plants samples can be attributed to the presence of secondary metabolites detected in the samples. It is noteworthy to make a comparative assessment of the effects of the extracts and the antibiotic used as standard. The control drugs had MIC and MBC values lower than those of the extracts. The antibiotic, though of lower concentrations, is synthetic in nature and expected to show more potency as it is reflected in the zones of inhibition. The effect of the antibiotic as regards the zones of inhibition, however, is not significantly higher than the extracts. The various conditions to which the extracts were subjected during the study could possibly have a direct effect on their antimicrobial activity. The observations above support the use of *C. pentandra* and *Cordia sebestena* in herbal cure remedies, and suggest that these plants may turn out to be a good replacement for the antibiotics in the future with further research focusing on the isolation of the active principle(s) of the plants.

Conclusion

The present study has investigated the *in vitro* antimycobacterial activities of the methanolic and dichloromethane extracts of the stem bark and leaf of the plants *C. pentandra* and *C. sebestena* and compared them with standard antimicrobial agent Rifampicin. The choice of the plants used in the study was based on their reported activities in the treatment of various disease conditions especially diseases resulting from infections of the respiratory tract. The organisms employed in this study were *M. fortuitum* ATCC 684*, M. smegmatis* ATCC 19420*, M. abscessus* and *M. phlei* ATCC 19240*.* All methanol extracts of the stem bark and leaf of the two plants exhibited antimicrobial activities on all the tested organisms, though at varying concentrations due to different concentration and composition of bioactive substances, while the dichloromethane extracts of the plants demonstrated little or no activity.

The inhibitory activities reported in this research have established the antimicrobial properties of *C. pentandra* (L.) Gaertn. and *C. sebestena* Linn**.** The antimicrobial activities of the extracts of the various parts of the plants against the organisms suggest that they can be used in the treatment of infections for which the organisms have been implicated. This justifies the ethnomedicinal use of the plants in the treatment of respiratory infections and associated diseases.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Effect of adding chlorhexidine to calcium enriched mixture (CEM) on its antimicrobial activity

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In recent years, a new endodontic cement, calcium enriched mixture (CEM) has been introduced; with clinical applications similar to those of mineral trioxide aggregate (MTA). It has been shown that CEM has higher antibacterial activity than MTA. On the other hand, use of chlorhexidine (CHX) to promote the antibacterial activity of different dental materials is increasing. The aim of the present study was to evaluate the effect of adding CHX to CEM on its antibacterial activity. The antibacterial activities of the materials under study [(CEM cement + CEM solution + 2%CHX) and (CEM cement + CEM solution)] against *Pseudomonas aeroginosa*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli* were evaluated using agar diffusion technique, followed by determination of the diameter of microbial zone of inhibition around the materials by three independent observers after 72 h. Data were analyzed by Mann-Whitney U test. Statistical significance was defined at P<0.05. The mean diameters of zones of inhibition in the CEM + CEM liquid and CEM + CEM liquid + CHX groups against *P. aeroginosa*, *E. faecalis*, *S. aureus* and *E. coli* were (13.2 and 9), (21.10 and 6), (20.2 and 9) and (17 and 9.75) ml, respectively, with larger diameters in the CEM + CEM solution + CHX group as compared to CEM + CEM solution group with all the microorganisms (P<0.05). Incorporation of CHX with CEM resulted in an increase in antimicrobial activity of CEM.

Key words: CEM cement, chlorhexidine, antibacterial.

INTRODUCTION

Mechanical pulp exposure and exposures due to caries in teeth with immature apices, without the symptoms and signs of irreversible pulpitis, should be sealed in order to preserve pulp vitality and prevent pathologic changes in periradicular tissues. In addition, communication pathways between the root canal and the periodontium, including perforations, should be sealed with restorative materials to prevent bacterial leakage. Since these

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materials are at close contact with vital tissues they should be biocompatible and induce regeneration of the affected tissues and restore the conditions before their involvement (Torabinejad and Walton, 2009). Mineral trioxide aggregate (MTA) is the most commonly used material to this end in the field of endodontics. The main ingredients of MTA are calcium oxide, sulfur tricalcium, phosphate oxide, silicon oxide, aluminum oxide, sodium oxide, manganese oxide and chlorine, which are mixed with a water-based liquid to yield bioactive calcium and phosphate (Asgary et al., 2008). Calcium enriched mixture (CEM) cement is a new dental material with applications similar to those of MTA. The results of a recent study showed that CEM cement releases calcium and phosphate ions (Asgary et al., 2008). CEM cement has a pH value similar to that of MTA; however, it has higher fluidity as compared to MTA with shorter working time and less film thickness (Asgary et al., 2008). A study by Asgary et al. (2008) showed that CEM has antimicrobial activity against pathogens, similar to that of calcium hydroxide and better than that of MTA (Asgary and Kamrani, 2008; Zarrabi et al., 2009). Antifungal effects of MTA and CEM against *Candida albicans* have been compared and it has been shown that both materials completely destroy the fungus in 24 h

Chlorhexidine has been initially introduced as an irrigation solution due to its broad-spectrum antibacterial activity (Barrios et al., 2013). Studies have shown that CHX is effective against bacterial species that are isolated from the infected root canals; these microorganisms include *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus salivarius*, *Esherichia coli* and *Candida albicans* (Ayhan et al., 1991; Nowicki and Sem, 2011).

Use of CHX to promote the antimicrobial properties of dental materials with the aim of improving prognosis is increasing. Studies have shown that adding 0.12% CHX to MTA increases its antibacterial activity (Stowe et al., 2004; Holt et al., 2007).

The aim of the present study was to evaluate the antimicrobial activity of CEM on *P. aeroginosa*, *E. faecalis*, *S. aureus* and *E. coli* using agar diffusion technique.

MATERIALS AND METHODS

The antimicrobial activities of the materials under study were evaluated against *P. aeroginosa*, *E. faecalis*, *S. aureus* and *E. coli* using the agar diffusion technique. Standard microbial strains were provided by the Department of Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences. All the bacterial strains were grown in Mueller-Hinton Broth (MHB) for 24 h at 37°C. Then a suspension was prepared from each bacterial strain at a concentration of 1.5×10^8 CFU/ml (turbidity equal to McFarland's 0.5 standard solutions). Each suspension was used to culture bacterial species on MHA using a sterile swab.

The materials under study were placed on the basal layer in each plate in a well. The plates were incubated at 37°C for 24 h. A total of eight plates were used for each bacterial strain, that is, on the whole 34 plates were used, which were randomly divided into four groups and two plates were used as positive and negative controls, containing solutions with and without microorganisms, respectively. Evaluations were carried out in sterile MHA culture media measuring 4 mm in depth in plates measuring 2 × 10 cm. A sterile punch was used to produce two identical holes measuring 4 mm in diameter at least 3 mm apart from each other in the basal layer of each plate. Each hole was filled separately with the materials under study, which consisted of the following: a mixture of 1 g of CEM cement powder + 0.36 mL of CEM cement solution, and a mixture of 1 g of CEM cement powder + 0.18 mL of CEM cement solution + 0.18 mL of 2% CHX (Conseppsis, Ultradent Products, South Jordan, Utah, USA). Finally, the diameter of zone of inhibition around each test material was measured using a ruler accurate to 0.5 mm, after 72 h.

Data were analyzed using descriptive statistics (mean ± standard deviation) and Mann-Whitney U test was used to compare means with SPSS 17. Statistical significance was set at $P<0.05$. Kolmogorov-Smirnov test was used to evaluate normal distribution of data.

RESULTS

With all the microorganisms under study, the mean diameters of zones of inhibition in the CEM + CHX group were significantly greater than those in the CEM group ($P<0.05$). Table 1 shows the mean diameters of zones of inhibition in the study groups.

DISCUSSION

In the present study, the antibacterial activity of CEM mixed with chlorhexidine was evaluated against *P. aeroginosa*, *E. faecalis*, *S. aureus* and *E. coli*. The results showed the positive effect of adding CHX to CEM on its antimicrobial activity.

Treatment of teeth with immature apices and repair of perforations are two important procedures in the field of endodontics and MTA is the most commonly used material to this end. CEM was introduced by Asgari et al. (2008) in recent years. The main constituents of CEM are calcium oxide, sulfur tricalcium, calcium phosphate, calcium carbonate, calcium silicate, calcium hydroxide and calcium chloride. CEM has dental applications similar to those of MTA (Asgary et al., 2008).

Studies comparing these two materials have shown that they have comparable sealing ability; however, the antibacterial activity of CEM is higher than that of MTA (Asgary et al., 2008).

Since microorganisms are the main factors involved in the failure of endodontictreatment, the antimicrobial activity of materials used in endodontic treatment has always been of great significance. The bacterial species included in the present study are real endodontic pathogens, which are related to cases resistant to treatment (Sundqvist, 1992). Although aerobic bacteria or the related microorganisms do not have a great role in initiating primary infections, they are found with a high frequency in root canal treatment failure cases (Siren et

Table 1. The mean diameters of zones of inhibition (mm) in the study groups.

Bacterial species	Mean diameter of zones of inhibition	
	CEM + CHX	CEM
E. Faecalis	21.10±0.86	6±0.43
P. aeroginosa	13.20±0.47	9±0.76
S. aureus	20.20±0.09	9±0.8
E. coli	17±1.61	9.75±0.6

al., 1997). These bacteria can enter the root canal system before treatment, during treatment and after treatment to cause secondary infection (Saunders and Saunders, 1994).

In the present study, agar diffusion technique was used, which is the most commonly used method to evaluate antibacterial activity (Torabinejad et al., 1995) and has been used by many researchers in a large number of studies (Kayaoglu et al., 2005).

Use of CHX is on the rise to increase the antibacterial activity of dental materials to improve prognosis. CHX is a synthetic cationic bis-guanide, which consists of 2 similar circles of 4-chlorophenyl and two bi-guanide groups, which are connected to each other with a central chain of hexa-methylene (Greenstein et al., 1986; Barrios et al., 2013). CHX is a lipophilic and hydrophobic positively-charged molecule, which reacts with bacterial cell membrane phospholipids and lipopolysaccharides and enters the cell through a number of active and passive transport mechanisms (Athanassiadis et al., 2007). Its action is attributed to the reaction of its positive charge with the negative charge of phosphate groups on the cell membrane (Gomes et al., 2003). Therefore, the osmotic balance of the cell is disrupted, increasing cellular permeability and allowing CHX to enter the bacterial cell. CHX is a base and is stable like a salt. The most commonly used oral form of CHX is its gluconate form, which is soluble in water and at physiologic pH releases positively charged CHX (Greenstein et al., 1986; Balla et al., 2013). At low concentration of 0.2%, low-molecular-weight components such as potassium and phosphorus exit the cell. On the other hand, at concentrations higher than 2%, CHX results in cell death (Gomes et al., 2003).

The results of the present study showed that adding 2% CHX to CEM solution results in a significant increase in its antibacterial activity, consistent with the results of a study carried out by Bidar et al. (2012) in which direct contact method and bacterial species other than those used in the present study were used. The antibacterial effect of CHX against all the microorganisms in the present study has already been shown (D'Arcangelo et al., 1991). Studies have shown that CHX is effective against bacterial species found in infected root canals, including *S. aureus*, *E. faecalis*, *S. salivarius*, *E. coli* and *C. albicans* (Ayhan et al., 1999).

Of course it should be kept in mind that adding CHX to WMTA results in cell death (Hernandez et al., 2005) and decreases its compressive strength (Holt et al., 2008). In addition, a mixture of MTA and CHX gel did not set for the last seven days (Kogan et al., 2006). On the other hand, the solution and gel forms of CHX exert different effects on the setting time of MTA. Kogan et al. (2006) mixed MTA powder with CHX gel in order to evaluate the compressive strength of this mixture; however, since the mixture did not set up to seven days after mixing, it was not possible to measure its compressive strength. Therefore, it is suggested that further studies should be carried out on CEM to evaluate the effect of adding CHX on its physical properties, such as compressive strength, setting time and sealing ability.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Characterization of cellulase producing from *Aspergillus melleus* by solid state fermentation using maize crop residues

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The production of cellulase by the yellowish orange sclerotia producing species, *Aspergillus melleus* UPAR01 on lignocellulosic material by solid state fermentation (SSF) was investigated. The first experiment was conducted to find out the colony radial growth rate (*Kr*) of fungus on solid medium using potato dextrose agar (PDA) and incubated at 30°C in the dark. The result shows that the average *Kr* value of *A. mellues* strain approximately was 0.77±0.03 cm/day. When the fungus was used to produce cellulase using maize crop residues as the sole carbon source by SSF at 30°C for seven day, the values of FPase, endoglucanase, β-glucosidase, and xylanase were achieved at 0.284±0.04, 9.45±0.33, 1.20±0.12, 12.58±0.08 U/mg protein, respectively. The optimal pH and temperature (°C) for the enzymatic activities was expressed by response surface methodology (RSM). The data shows that the optimum pH range was between 5.5 and 5.8 and the optimum temperature ranged from 53 to 59°C. In addition, none of the metal ions and ethylene-diaminetetra-acetic acid (EDTA) induced cellulase and xylanase activities.

Key words: *Aspergillus melleus*, cellulase, solid state fermentation (SSF), maize crop residues.

INTRODUCTION

Cellulase is a group of enzymes that synergistically work to hydrolyze cellulose to glucose. It is composed of endoglucanase (endo-1, 4-β-D-glucanase, EC 3.2.1.4), exoglucanase (1,4-β-D-glucancellobiohydrolase, EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucanohydrolase or cellobiase, EC 3.2.1.21) (Joo et al., 2009; Gao et al., 2008). Although xylanase (EC 3.2.1.8) is not part of the cellulase system but this enzyme is needed in the hydrolysis of lignocellulosic biomass which provides an appreciable amount of hemicellulose or xylan (Kamble and Jadhav, 2011).

Solid state fermentation (SSF) of lignocellulosic material for production of cellulase and xylanase is an attractive means to produce enzymes because of its

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lower capital investment and lower operating cost. Several studies have indicated that agricultural residues including maize crop residues can be used as lignocellulosic carbon source for the production of cellulase (Liming and Xueliang, 2004; Zhu et al., 2009; McMillan et al., 2011) including solid state fermentation of castor bean (*Ricinus communis* L.) for cellulase production by *Aspergillus japonicas* URM5620 (Herculano et al., 2011). Both bacteria and fungi are known to produce cellulase, however, fungal enzymes are generally complete comprising all the cellulosic activities (Stockton et al., 1991). *Aspergillus* species are well known to be good for producing cellulases and xylanase and many species have been studied, including *A. terreus* (Gao et al., 2008), *A. niger* (Ncube et al., 2012), *A. fischeri* (Senthikumar et al., 2005), *A. niveus* and *A. ochraceus* (Betini et al., 2009).

The aims of this experiment were to investigate the enzymes kinetics including optimal pH, temperature and kinetics based on the optimization by Response Surface Methodology (RSM).

MATERIALS AND METHODS

Fungal cultures

The fungus *Aspergillus melleus* UPAR01 used in this investigation was originally isolated from maize crop area, Phayao province, Thailand (2010) using modified *Aspergillus flavus* and Parasiticus Agar (AFPA) (Cotty, 1994). The culture was transferred and preserved in Potato Dextrose Agar (PDA) slants at 4°C in the culture collection of the Biotechnology program, School of Agriculture and Natural Resources, University of Phayao, Thailand. To prepare spore suspension, *A. melleus* fully grown spores (approximately seven days incubation on PDA at 30°C) were suspended in 0.85% (w/v) sodium chloride to prepare the homogenous spore suspension (10^6 spore/ml).

Media preparation

Potato Dextrose Agar (PDA) and Malt Extracted Agar (MEA) were purchased from Difco, USA. V8 medium (Cotty and Misaghi, 1984) consisted of 5.0% (v/v) V8 juice and 2.0% (w/v) agar. Modified *A. flavus* and Parasiticus Agar (AFPA) (Cotty, 1994) consists of 1.0 %(w/v) peptic digest of animal tissue, 2.0 %(w/v) Yeast extract, 0.05 % (w/v) Ferric ammonium citrate, 1.0 ml of 0.2% (w/v) dichloran in ethanol and 1.5 % (w/v) agar. Semi-synthetic medium (SS) consists of 5.0 g of lignocellulose substrate followed with 40 ml of the SS medium in a 250 ml Erlenmeyer flask. The SS medium contains 0.2% (w/v) peptone, 0.1% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$, 0.01% (w/v) $CaCl_2$, 0.2% (v/v) Tween 80 and 0.5 ml of all each trace elements stock solution including 0.0005 % (w/v) $ZnSO_4.7H_2O$, 0.0005% (w/v) $FeSO_4.7H_2O$, 0.002% (w/v) and 0.005% (w/v) $MnSO_4.H_2O$. The pH of all media was adjusted to 5.5, and sterilized by autoclaving at 120°C for 15 min.

Substrate preparation

Maize crop residues (stalk, stover, and hub) were collected from Dok Kham Tai district, Phayao province, Thailand. Sample was dried in a hot air oven at 60°C for 72 h and then ground and passed through 2 mm sieve. The sample was analyzed for lignocellulosic compositions by the detergent method (Goering and Van Soest, 1970) and was used as substrate for enzyme production.

Characterization of *A. melleus*

A. melleus was identified according to taxonomic schemes proposed by Klich (2002). Macroscopic characteristics, including colony diameter, colony colors, colony texture, conidial color, sclerotia, reverse color, and soluble pigment, were observed by naked eye. Fine structural characteristics, including seriation, vesicle shape, stipe length, and the shape, size and texture of conidia, were observed under a dissecting microscope.

The molecular investigation was examined in the laboratory test. *A. melleus* was inoculated into Malt Extracted Agar (MEA) plate at 25°C for 7 days in the dark, and then its DNA was extracted from the culture using MoBio-UltracleanTM Microbial DNA Isolation Kit. Fragment containing part of the β-tubulin gene was amplified using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC) (Glass and Donaldson, 1995). The PCR fragments were sequenced with the ABI Prism® Big DyeTM Terminator v. 3.0 Ready Reaction Cycle sequenced Kit. Samples were analyzed on an ABI PHISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the programme SeqMan from the LaserGene package. For storage, isolates were grown on V8 medium (5% V8 juice and 2%agar) (Cotty and Misaghi, 1984), from which 3-mm plugs of sporulating culture were taken and placed in vials containing 5 ml of 0.01% Triton X-100 (TX).

Measurement of colony radial growth rate (*Kr*), mycelia agar plug (0.2 cm) of *A. melleus* was inoculated onto the center of 20 ml of PDA in Petri dish and then incubated at 30°C for 7 days in the dark. Fungus was measured for the radial growth of colonies (cm) by a ruler with repeated 3 times for 3 days. Typically, the fungal mycelia had grown and reached into the end of the plate approximately in 6 to 7 days. After the last measurements, the *Kr* was determined according to the method described by Morrison and Righelato (1974). The linear regression was $Kr = dr/dt$ in which dr/dt was colony radial growth rate (cm) obtained during $time_o$ (observed time) and $time_i$ (initial time), respectively.

Production of enzymes

Solid State Fermentation (SSF) process was used in this study. The maize crop substrate (5.0 g) was weighed and put into a 250 ml Erlenmeyer flask with 40 ml of the Semi-synthetic medium (approximately 63% moisture content using Extech SDL550 Moisture Content Meter, Datalogger, USA). Culture medium was inoculated with 2.0 ml of *A. melleus* UPAG01 spore suspension (10^6 spore/ml) and incubated at 30°C for 7 days. After incubation, crude enzymes were extracted by adding 50 ml of 0.01% (w/v) Tween 80 in 50 mM sodium citrate buffer pH 5.0 and mixed for 1 h on a rotary shaker at 150 rpm. The suspension was then centrifuged and the same buffer was added to the clarified supernatant to make a total volume 100 ml. The suspension contained the crude enzymes used for further studies.

Determination of enzyme activities

Cellulase (FPase, endoglucanase and β-glucosidase) and xylanase activities were assayed according to the method described by Ghose (1987) and Ghose and Bisaria (1987). The reaction mixtures were determined by incubating 0.5 ml of enzyme solution with 50

Table 1. Variables and their level for 2^2 factorial central composite designs for cellulase and xylanase activities (U/ml).

Sample number	Natural variables		Coded variables	
	pH*	Temp (°C)	χ_1	χ_1
1	4.0	45	-1	-1
2	4.0	75	-1	1
3	8.0	45	1	-1
4	8.0	75	1	1
5	3.172	60	-1.414	0
6	8.828	60	+1.414	0
7	6.0	38.74	0	-1.414
8	6.0	81.21	0	+1.414
9	6.0	60	0	0
10	6.0	60	0	0
11	6.0	60	0	0
12	6.0	60	0	0
13	6.0	60	0	0

mM citrate buffer pH 5.0 containing one piece of 1X6 cm Whatman No.1 filter paper for FPase, 0.5 ml of 2.0 %(w/v) carboxymethylcellulose (CMC) for endoglucanase and 0.5 ml of 1.0% (w/v) xylan for xylanase, respectively. The reaction mixtures were incubated at 50°C for 60 min (FPase) and 30 min (endoglucanase and xylanase). β-glucosidase activity was assayed according to the method described by Sternberg et al. (1977). The reaction mixture was determined by incubating 0.5 ml of enzyme solution and 0.5 ml of 4 mg/ml D-salicin, followed by incubation at 50°C for 30 min.

After incubation, all reactions were assayed by estimating the reducing sugar liberated using DNS reagent (Miller, 1959), and were calculated in international units of enzyme activity. One unit of cellulase (FPase, endoglucanase, and β-glucosidase) is defined as the amount of enzyme that liberates 1.0 µmol of glucose per 1.0 min under the assay conditions. One unit of xylanase is defined as the amount of enzyme that liberate 1.0 µmol of xylose per 1.0 min under the assay conditions.

Lowry method (Lowry et al., 1951) was used in this study for protein determination. The amount of total proteins (mg/ml) was performed by means of standard curve obtained from solutions containing (0.0-2.0 mg/ml) bovine serum albumin (BSA).

Kinetic characterization (pH and temperature) by response surface methodology effect of pH and temperature

Sodium citrate (50 mM) buffer pH 3.172, 4.0 and 6.0 and 50 mM sodium phosphate buffer pH 8 and 8.8 were examined in this study. The statistical approach with Response Surface Methodology (RSM) was used to determine the optimal conditions to obtain the highest activity of enzymes. The experiments were based on 2^2 full factorial Central Composite Design (CCD) with augmented points in two independent variables including χ_1: pH, and χ_2: temperature values, while enzyme activity (γ; U/ml) was the dependent variable as described in Table 1.

All measurements were performed in three replicates, and data were expressed as average values, and analyzed using Minitab 5.1 software. The lower *p*-value and insignificant lack of fit was obtained with a quadratic model that suggested good fit. Higher coefficients of regression (95%) suggested that there was good agreement between predicted and estimated enzymatic activities. The results of RSM were used to fit a second-order polynomial equation that represents the behavior of the system; $\gamma = \beta_0+\beta_1\chi_1+\beta_2\chi_2+\beta_{11}\chi_1^2+ \beta_{22}\chi_2^2+\beta_{12}\chi^1\chi^2$

Effect of metal ions

The effects of metal ions which included Mg ($MgSO_4$), Ca ($CaCl_2$), Mn ($MnSO_4$), Fe($FeSO_4$), Co($CoCl_2$), Cu($CuSO_4$), Zn($ZnSO_4$), and ethylene-diaminetetra-acetic acid (Na-EDTA) were investigated by incorporating them to 0.1 M final concentration mixture prior to determination of enzyme residual activities.

Km* and *Vmax

Four types of substrates, Avicel®, CMC, salicin and xylan were also chosen to test the substrate specificity of FPase, endoglucanase, β-glucosidase and xylanase, respectively. After the selection of the optimum pH for enzyme activities, the Michalis-Menten for *Km* and *Vma*x values were determined by the investigation using varying substrate concentrations ranging from 0 to 0.25 %(w/v), and the assays were conducted in the same manner of cellulases and xylanase determination.

RESULTS AND DISCUSSION

The AFPA medium was modified by Pitt et al. (1983). This medium was used for *Aspergillus* spp. isolation by several authors including Reddy et al. (2009) and Foley et al. (2014). The medium was used to select *Aspergillus* sp. including *A. flavus* or *A. parasiticus* because both fungi produced yellow orange color when viewed from the reversed side of the plate. Yellow orange color of colony was observed because medium help improved color production on the reverse of the plate due to the optimal concentration of a more soluble iron salt and the addition of yeast extract (Pitt et al., 1983). In this study, *A. melleus* UPAG01 color change of the reverse phase on AFPA was similar as *A. flavus* or *A. parasiticus* by appearance of yellow orange color reverse colony. Conidia and mycelium germination of isolates *Aspergillus* could be observed in these AFPA medium except groups of *A. nomius.* Damann et al. (2004) showed AFPA medium did not support sporulation by *A. flavus*. *A. melleus* showed dark yellow reverse colony similarity as *A. flavus* and Foley et al. (2014) reported that *Aspergillus* spp. produced yellow orange color when viewed from the reversed side of the plate. However, *A. melleus* UPAG01 produced yellow radiate conidia heads (50-150 µm) which enable clear differentiation from *A. flavus* or *A. parasiticus* (green conidia). The microscopic morphology showed that the hyphae (2-3 µm diameter) were septate, hyaline, and biserate with smooth wall globose conidia (2-3 µm diameters) which was reported by Christensen (1982) and Klich (2002). In addition, this strain produced abundantly yellow to brown sclerotia on 5% V8 medium and PDA (Klich, 2002) (Figure 1). When *A. melleus* UPAG01 DNA was extracted, to identify the species of

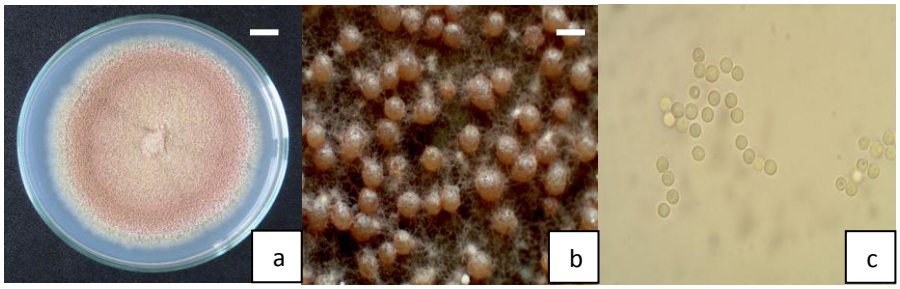

Figure 1. Morphological characteristics of *A. melleus* UPAG01 (a) conidia (pale yellow) and sclerotia (dark brown) on PDA (bar=1 cm)(b) scerotia on PDA (bar=0.1 mm) (c) Conidia under microscope.

Table 2. Equations analysis for optimal pH and temperature (coded values).

Enzymatic acticity (U/ml); γ	Fitted Equation	pH optimum (χ_1)	Temperature (°C) optimum (χ_2)
FPase; γ_1	$0.1872-0.013\chi_1-0.010\chi_2-0.061\chi_1^2-0.066\chi_2^2$	-0.059	-0.130
Endoglucanase; γ_2	$1.538-0.262\chi_1-0.401\chi_2-0.250\chi_1^2-0.400\chi_2^2$	-0.832	-0.220
β–Glucosidase; γ_3	$0.145-0.016\chi_1-0.017\chi_2-0.030\chi_1^2-0.035\chi_2^2$	-0.246	-0.70

Aspergillus was followed according to the method described by Glass and Donaldson (1995). The sequences were compared on GenBank using BLAST and in a large fungal database of CBS-KNAW Fungal Biodiversity Centre with sequences of most of the type strains. The results had a 100% match in all databases with the type strain of *A. melleus* sample that is a fungus in the Subgenus *Circumdati*, Section *Circumdati*.

For the measurement of colony radial growth rate (*Kr*), several reports showed that *Aspergillus* sp. had wide range of *Kr* values that depended on species, strains and culture conditions. The *Kr* values of *Aspergillus* strains isolated from honey bee and *A. flavus* ATTC 16872 were 0.228 and 0.24 cm/day, respectively (Yoder et al., 2008; Lopez-Malo et al., 1998). High or low values of pH and chemical substances which included bicarbonate, vanillin and avermectin (more than 0.01% w/v) affected colonies formation by *Aspergillus*, and lead to the reduction of the values of *Kr* (Lopez-Malo et al., 1995; Rodr´ıguez-Urra et al., 2009; Danmek et al., 2011). In this study, PDA with pH at 6.0 was used because several reports discussed PDA as suitable for the culturing of *Aspergillus* and had no effect in the range between pH at 5.5 to 7.5 (Brancato and Golding, 1953; Cotty, 1988; Thompson, 1990; Yoder et al., 2008). In addition, Gupta et al. (2012) showed that PDA is the most commonly used as laboratory medium for fungi due to its good and balanced nutrient content. *A. melleus* UPAG01 grew well and produced white mycelia and brown conidia with dark-brown sclerotia until the end of the PDA plate within 6 days at 30°C in the dark same as the other *Aspergillus* spp. including *A. flavus* and *A. niger* (Reddy et al., 2009; Foley et al., 2014). Data showed the beginning of exponential phase at the second day of incubation. Therefore, linear regression of *A. melleus* UPAG01 was calculated using the relationship between colonies radial growth (cm) versus time (day) that at this time. Average *Kr* value of all *A. melleus* UPAG01 for 6 days was 0.77±0.03 cm/day.

Among the selected maize crop residue, it contained the high cellulose (33.15%dw) hemicellulose (44.67%dw) and low lignin content (4.91 %dw). The residue was selected as a carbon source for enzyme suitable for production of cellulase and xylanase by *A. melleus* UPAG01. Under acidic conditions with pH 5.5 and 7 days of incubation, *A. melleus* UPAG01 produced 0.28±0.04, 9.45±0.33, 1.20±0.12 and 12.58±0.08 U/mg of FPase, endoglucanase, β-glucosidase, and xylanase, respectively. The high yield might result from its lignocellulosic materials present in inducers and nutrients. Several reports indicated that cellulase and xylanase production were favored in the acidic range of pH 4 to 6 (Brijwani et al., 2010; Romero et al., 1999; Tishkov et al., 2013). Similarly, Pothiraj et al. (2006) and Mrudula and Murugammal (2011) reported that *A. terreus* and *A. niger* produced high value of cellulase activities in the sixth to eight day of incubation by Solid State Fermentation (SSF). For optimal pH and temperature, the overall second order polynomial equation for the activities as measured in term of Unit (U/ml) is shown in Table 2. The three dimensional plots of the combined parameters between χ_1 and χ_2 on the enzyme activities are shown in Figure 2. The negative significant coefficient of χ_1, and χ_2 values are expressed as a linear effect; this is meant for decreasing enzyme activities (γ).

For the other terms, two quadratic terms of χ_1^2, and χ_2^2

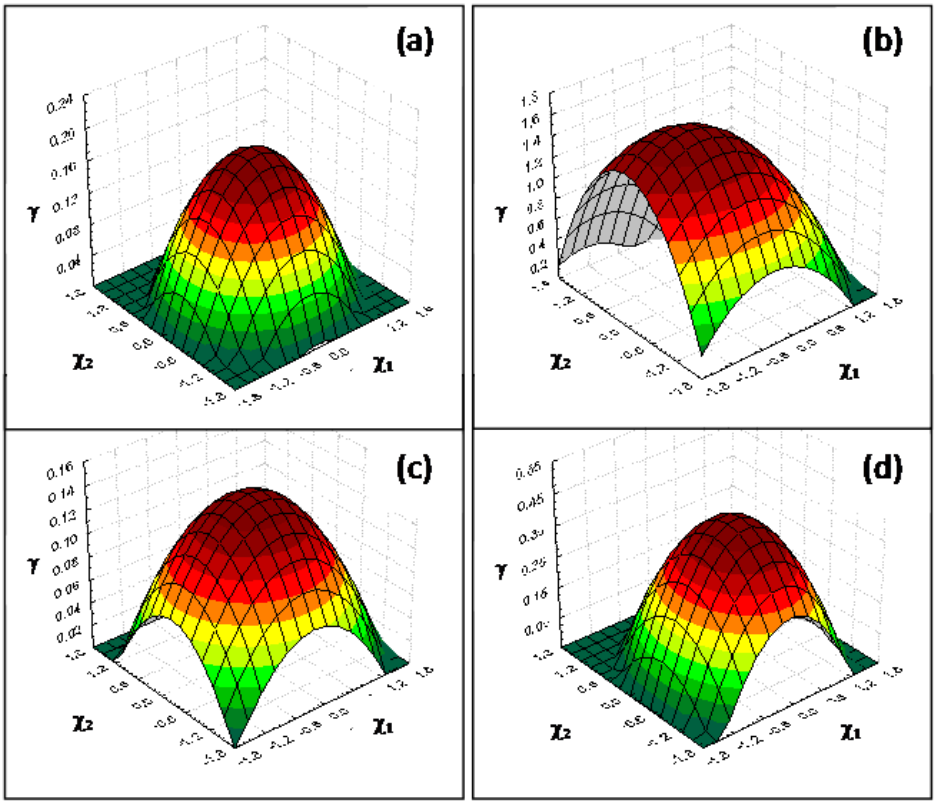

Figure 2. Three dimensional plots of the combined effects between pH (χ_1) and temperature (χ_2) on enzyme activities (γ; U/ml) from *A. melleus* UPAG01: FPase (a), endoglucanase (b), β-glucosidase (c), and xylanase (d)

were found to be expressed similarly as linear effect whereas interaction term ($\chi_1\chi_2$) was not significant. The optimal pH and temperature range for cellulase and xylanase activities production by *A. melleus* UPAG01 were found to be at pH 5.5 to 5.8 and at temperature of 53 to 58°C which was similar as the other *Aspergillus* species including *A. terreus* (Gao et al., 2008), *A. fumigatus* (Das et al., 2013) and *A. oryzae* (Riou et al., 1998; Hoa and Hung, 2013) when using the Equation in Table 2, respectively. The results of interaction between the temperature and pH revealed that increasing both temperature (>60°C) and pH (>6.0), leads to a decrease of enzyme activity. Under strong acidic (pH 3.17) and alkali conditions (pH 8.82) at high temperatures (>75°C), the enzyme activities were much lower. Other reports mentioned that the optimal pH and temperature for cellulase and xylanase activities from the other fungi, *A. terreus* (Gao et al., 2008), *A. fumigatus* (Das et al., 2013) and *A. oryzae* (Hoa and Hung, 2013), were in the pH range between 4.5 to 6.0 and temperature range between 50 to 60°C.

The effect of metal ions and substances on cellulase and xylanase of *A. melleus* UPAG01 are summarized in Figure 3. None of the metal ions and substances induced both cellulase and xylanase activities. The high values inhibitions of all enzymes were found in the presence of Cu and EDTA similarly as the results obtained by Tejirian and Xu (2010) and Quay et al. (2011). Approximately 50% of relative activities were found in the present of Cu ion (52±2.96% in FPase, 57±5.23% in endoglucanase, 48±0.71% in β-glucosidase and 46±0.75% in xylanase, respectively). The inhibition of enzymes by Cu ion might be affected by the high Cu concentration (0.1 M final concentration). However Tavares et al. (2013) showed that cellulase from *Aspergillus* spp. including *A. nidulan* could be induced when using low and optimal Cu concentration (0.018 M). In addition, Cu and EDTA ion (0.015 mM), activated cellulase activity (endoglucanase) from *Aspergillus awamori* VTCC-F099 by up to 55% (Nguyen and Quyen, 2010).

Crude *A. melleus* UPAG01 enzymes appears to be a good candidate, which is very useful for their applications to degrade lignocellulosic material. The relationship between enzyme activities and substrate concentrations produced typical Michaelis-Menten curves. *Km* values were found to be 0.09 (FPase), 0.668 (endoglucanase), 0.069 (β-glucosidase), and 2.176 g/L (xylanase) whereas *V*max values were found to be 0.003 (FPase), 0.044 (endoglucanase), 0.083 (β-glucosidase), and 1.071 $\mu mol.min^{-1}.ml^{-1}$ (xylanase) when each Avicel®, CMC,

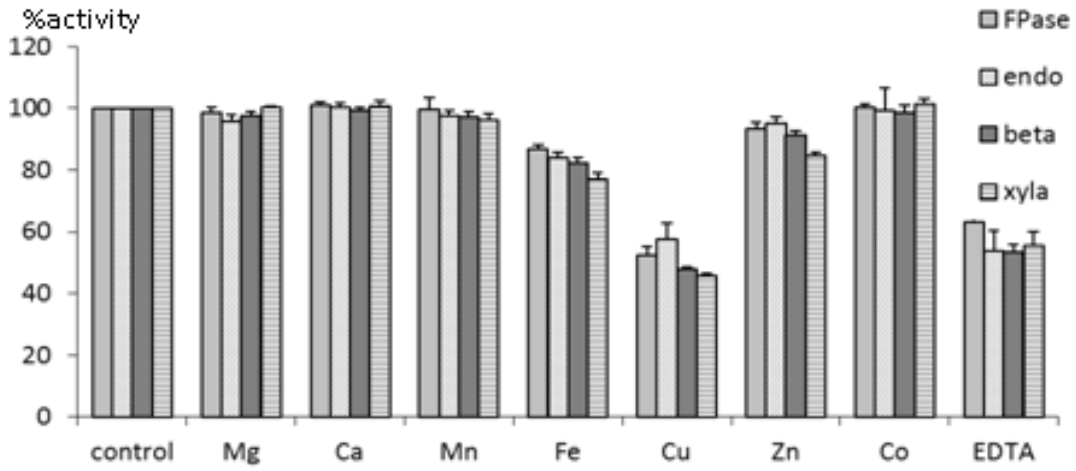

Figure 3. Effects of metal ions and Ethylene Diamino Tetraacetic acid (EDTA) on the activity of the enzymes from *A. melleus* UPAG01.

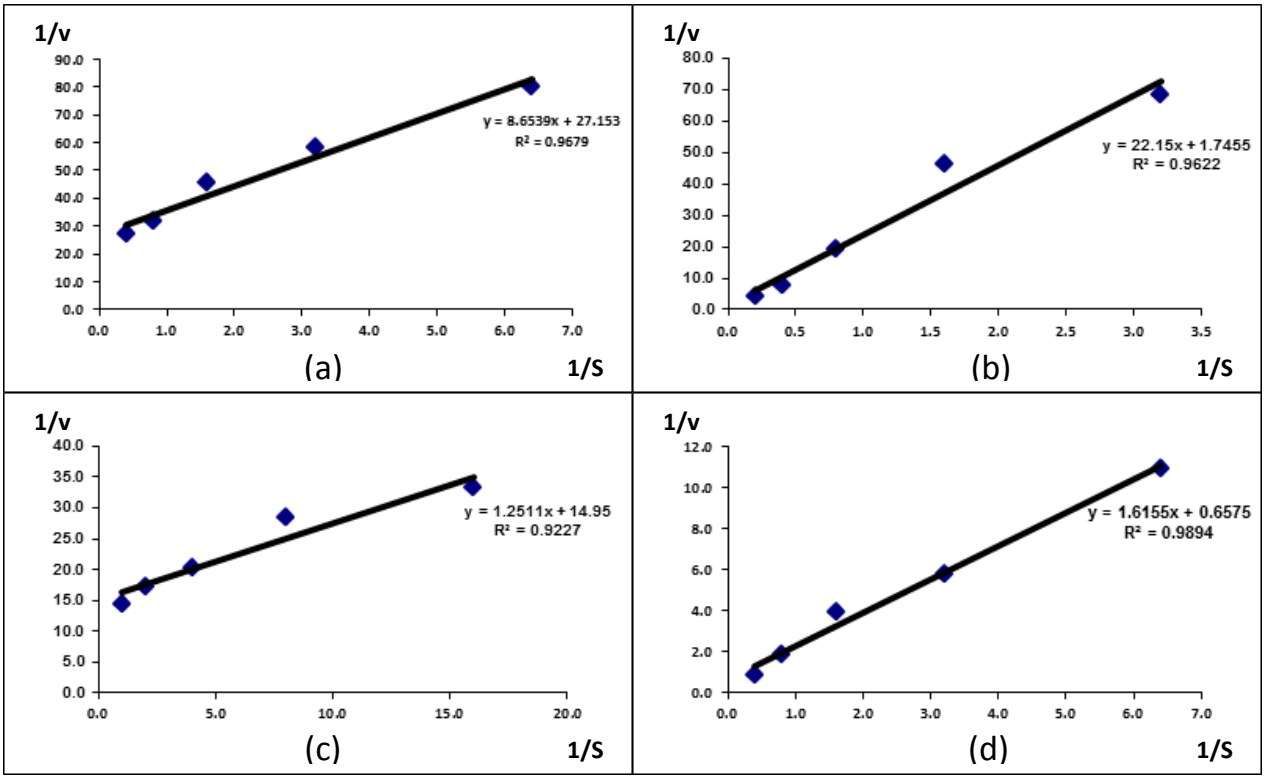

Figure 4. Michaelis-Menten curves of *A. melleus* enzymes determined by the investigation using varying substrates including Avicel® (a), CMC (b), salicin (c) and xylan (d).

salicin, and xylan was used as the substrate (Figure 4). For this observation, selection of the appropriate storage solvent (sodium citrate) and specificity with substrates seems to be crucial for cellulase and xylanase in order to maintain high levels of enzymes for either storage and in biodegradation reactions of lignocellulosic materials.

Conclusion

According to the investigation, the use of the lignocellulosic material, maize crop residue, as the sole carbon source was a candidate for the production of cellulase and xylanase. The unique enzymes from *A. melleus* could be used for hydrolysis of local agricultural biomass.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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